HER2, P53, FAS, FASL, COX2, PGE_2S, EGFR EXPRESSION IN BREAST CANCER AND IN NORMAL PERITUMORAL BREAST TISSUE: POTENTIAL NOVEL RISK BIOMARKERS

EDIT NÁDASI M.D.

TUTOR:
ISTVÁN EMBER M.D., PH.D., D.SC.

2004

CONTENTS

SUMMARY

Breast cancer is the most common cancer among women in many parts of the world and early detection is the key to the survival of the patients. There is evidence that changes in HER2 and p53 protein expression might be relevant to breast cancer progression. Furthermore, we have recently reported that malignant breast tumors show an altered expression of Fas and Fas ligand (FasL) compared to normal tissues and these molecular changes are significantly related to patient outcome and COX2 is a relevant new prognostic marker. In this study we hypothesized that these molecular markers might also be useful to evaluate the malignant potential of non-neoplastic breast tissues.

To this end, we analyzed, by using specific antibodies, HER2, p53, Fas and FasL expression in 72 breast carcinomas, the corresponding autologous peritumoral tissues (PTT) sampled at 1, 2 and 3 cm far from the tumor itself and in 44 benign mammary lesions. Ten breast carcinomas and their autologous 1 cm PTT samples were also analyzed by fluorescent in situ hybridization (FISH) to determine if HER2 gene amplification can be demonstrated in the background of cytoplasmic immunohistochemical HER2 staining. Further 186 stage I-II primary BC and 95 autologous metastatic lymphnodes were analyzed immunohistochemically for COX2, hormones receptor, p53, HER2, Fas and Fas ligand (FasL) expression to determine the effect of COX2 expression on the prognosis of breast cancer patients receiving chemotherapy either as a single factor or taken into consideration together with other factors. To understand better the probable role of functional COX2 alteration in developing primary breast cancer and metastases, we also analyzed the expression of PGE_2S in 121 primary breast tumors and of epidermal growth factor receptor (EGFR) in 167 of the cases Because of insufficient quantity of tissue samples no complete analyses for all the 186 cases and their 95 metastases were available.

Results obtained suggest that HER2 gene amplification often underlies even cytoplasmic HER2 staining when analyzed immunohistochemically. Furthermore, breast carcinomas and the closest adjacent uninvolved parenchyma shared an upregulated FasL phenotype which is lost in PTT farther from the tumor. Therefore, among the biological parameters investigated, HER2 and FasL expression seems to represent biomarkers of breast tumorigenesis easily applicable to fine needle aspirates and potentially useful to detect patients at high risk of breast carcinoma. Moreover, in high-risk breast cancer patients the immunohistochemical evaluation of COX2, together with PGE_2S, p53, Ki67, HER2, Fas and FasL, may be of clinical value in distinguishing different responses to adjuvant anthracycline-based chemotherapy.

1. INTRODUCTION

About 180.000 new cases of breast cancer (BC) are diagnosed yearly and despite improvements in screening programs and integrated treatments, mortality from this cancer has only moderately decreased, suggesting that this malignancy, at diagnosis, is often a systemic disease. Therefore early detection is, at the present, the key strategy to improve the outcome of patients bearing this neoplasia. The development and progression of BC, as in other solid tumors, result from the accumulation of genetic alterations. Therefore it is likely that some types of benign lesions (BL), precursors for invasive BC and the normal appearing peritumoral tissue (PTT), according to the field cancerization hypothesis, may harbor molecular changes representing signatures of clinical relevance which heralds early stages of cancer development. In BC this hypothesis is becoming increasingly supported by experimental data. At genetic level, it has been reported that the frequency of chromosomal abnormalities found in benign tumors, although lower than in BC, correlates with the corresponding risk of developing invasive carcinoma (1). Moreover, the benign parenchyma of cancer-containing breast and the contralateral epithelium in patients who experienced cancer in one breast, can share with invasive carcinoma the same pattern of chromosomal abnormalities (2).

The analysis of tumor suppressor genes has demonstrated that p53 gene mutation and protein nuclear accumulation can be detected in benign breast lesions (3, 4) as well as in normal breast epithelium adjacent to excised tumor (5, 6). These findings suggest that perturbations in p53 function may occur in breast tissue before morphological changes are apparent. Although it is often assumed that somatic p53 mutations not altering coding may be silent, such alterations could still serve as an index of accumulating genetic damage and/or defects in DNA repair.

The scrutiny of changes in expression pattern of oncogene products associated with unfavorable prognosis has revealed that overexpression of HER2 (7) is an early event in breast carcinogenesis since it is detected in a higher percentage of in situ carcinoma than of the invasive one (8). Furthermore, HER2 gene amplification and protein overexpression can be found in benign proliferative breast lesions such as typical (TDH) and atypical ductal hyperplasia (ADH) and in morphologically normal appearing mammary epithelium adjacent to invasive cancer (6, 9, 10). Cell cycle control is a highly complex finely tuned process in which genes modulating cell proliferation i.e. p53, HER2 are balanced by gene products controlling apoptosis such as the Fas-FasL system. In this context we have reported that benign and malignant mammary lesions are characterized by differential expression of this

complementary receptor/ligand antigen and that a tumor with a $Fas^-/FasL^+$ phenotype is associated with an unfavorable outcome (11). Because Fas is more homogeneously expressed in benign than in malignant tumors (11, 12, 13) and BRCA-1 associated cancers express higher level of FasL than the sporadic disease, changes in Fas/FasL phenotype might represent an early biomarker of transformation. To address this issue in the present study we extended the analysis of Fas/FasL expression to the normal appearing PTT. To this end we submitted to a parallel immunohistochemical (IHC) analysis, which included HER2, p53, Fas and FasL, 72 BC and multiple samples of the autologous normal appearing peritumoral epithelium sampled at 1, 2 and 3 cm from invasive cancer to determine whether a gradient of molecular alterations may occur.

Recent epidemiologic studies have indicated that prolonged use of nonsteroidal anti-inflammatory drugs (NSAID) is associated with a decreased risk of several malignancies, most notably colorectal cancers (14, 15, 16, 17). Similar relationships have been observed in lung, breast and other cancers as well (18, 19). Since NSAID, as a principal action, inhibit cyclooxygenases, these findings suggest that cyclooxygenase (COX) activity and thus prostaglandin (PG) synthesis contributes to the risk of developing primary cancers (20). High COX expression is a common feature of human epithelial malignancies (21, 22), however, the biological significance of this metabolic activity is not completely clear yet. Two isoforms of COX are known: COX1 is expressed ubiquitously, and its role has been connected to physiological functions such as maintenance of the gastric mucosa and regulation of the renal blood flow, whereas COX2 is induced as an immediate-early gene in most cells and its expression is not detectable in most healthy tissues, but can be induced in response to various extracellular stimuli, including growth factors, cytokines, tumor promoters, peroxisomal proliferators and carcinogens (16). Additionally, COX2 is responsive also to several oncogenes (23, 24, 25). Even though cancer cell lines express both COX isoforms, the majority of the PG synthesis - which may be inhibited by the NSAID administration - stems from the activity of the inducible COX2 isoform (22). COX2 is known to induce prostaglandin E_2 synthase (PGE_2S) activity (26) while synthesized PGE_2 is related also to the metastatic ability of the primary tumor (27). COX2 also induces epidermal growth factor receptor (EGFR) expression and thus mediates trophic actions on gastrointestinal mucosa in human colon cancer cell lines (28).

In contrast to colon cancer, the role of COX2 in breast cancer is less clear. Poorly differentiated histological features were found to correlate with low COX2 expression in mammary, pulmonary and colonic tumors. In breast carcinomas, in situ malignancies were

more likely to express COX2 than invasive carcinomas (29). Furthermore, in a murine model system positive correlation of both COX2 expression and metabolic activity to tumorigenic and metastatic potential was shown (30). It has also been revealed that high levels of COX2 are expressed in human mammary tumor tissues compared to the adjacent normal tissue (31), which may indicate an induced COX2 function due to the changes the malignant cells have undergone. Several studies have revealed an association between NSAID consumption (and thus inhibition of COX2 activity) and decreased breast carcinoma incidence (18, 32, 33), while others have failed to find a significant relationship between aspirin use and breast cancer risk (14, 34). The basis for this lack of consistency among different studies is unclear. Conflicting data obtained in separate studies may reflect the usage of different NSAID in the populations examined. Another potential explanation is that significant COX2 overexpression may be limited to a subset of human breast cancers (20).

To this end 186 stage I-II primary BC and 95 autologous lymph node metastases were analyzed immunohistochemically for COX2, hormones receptor, p53, HER2, Ki67, Fas and FasL expression to determine the effect of COX2 expression on the prognosis of breast cancer patients receiving chemotherapy. To understand better the probable role of functional COX2 alteration in developing primary breast cancer and metastases, we also analyzed the expression of PGE_2S in 121 primary breast tumors and of epidermal growth factor receptor (EGFR) in 167 of the cases. Because of insufficient quantity of tissue samples no complete analyses for all the 186 cases and their 95 metastases were possible.

1.1 AIMS

Since the development and progression of breast cancer, as in other solid tumors, result from the accumulation of genetic alterations, it is likely that some types of benign lesions, precursors for invasive breast cancer and the normal appearing peritumoral tissue, according to the field effect hypothesis, may harbor molecular changes representing signatures of clinical relevance which heralds early stages of cancer development.

The aims of our two studies were

1. to determine immunohistochemically the HER2, p53, Fas, FasL, hormone receptores, COX2, PGE_2S and EGFR expression in breast cancer tissues and in morphologically normal-appearing peritumoral tissue samples taken 1cm, 2 cm and 3 cm far from the tumors.
2. To determine HER2 gene amplification by FISH in HER2 positive 1 cm peritumoral tissue samples to support the field-effect hypothesis.

3. To determine the influence of the investigated markers on the 5-year disease-free survival and overall survival of the breast cancer patients.
4. To determine the combination of markers most useful to determine accurate prognosis.
5. To establish a set of markers for diagnosing high risk of recurrence by determining field-effect in peritumoral tissue samples.

1.2. HISTORY OF BREAST CANCER

The oldest record of breast cancer dates back to 1600 BC. This document is the oldest known medical record and supposed to be a transcript made in 1600 BC of a papyrus dating back to about 3000 BC (35).

Celsus realized the value of surgery in the early stages of the breast tumor and said that only small tumors could be removed, while larger tumors would only be irritated by surgical intervention. Hippocrate (460 AD) distinguished between benign and malignant tumors. He considered breast cancer incurable (36). According to the doctrines of Galen (130-200 AD), melancholia was the main factor in the development of breast cancer. Special diets and exorcism were the recommended treatments.

An English physician, Thomas Willis (1621-1675), used almost the same definition of tumor with respect to neoplasia as it is used today. According to him, tumor is the "disturbance of growing primary characterized cells with uncontrolled, non-purposefully dividing cells"

During the Renaissance, Andreas Vesalius recommended mastectomy as well as ligatures (sutures) to control the bleeding, rather than cauterization. The fact that breast cancer could spread to the regional axillary nodes was first recognized by the physician LeDran (1685-1770) who was probably the very first person in history to associate poor prognosis with the spread of breast cancer to the lymph nodes (37).

The first epidemiological data of breast cancer came from Middlesex Hospital in London (1791-1805), where the first breast cancer cases were registered. Two hundred-fifty of these patients refused treatment (37). Although anatomical knowledge improved in the 18th century, the outcome of breast surgery did not, due to infection, lack of good anesthesia, and the use of complete radical mastectomy. A new era of surgery and medicine began with the discovery of NO as an anesthetic in 1846, antiseptic techniques in 1867, and microscopic histological analysis.

Halstead and Meyer brought light to the ill-fortuned women diagnosed with breast cancer. In 1894 each of them independently announced his surgical procedures and results for treatment of breast cancer. They described, for that period of time, a superior local control of disease by

en-bloc radical mastectomy which included total removal of the affected breast, total ipsilateral axillary lymph node dissection in levels I-III, resection of pectoral major and minor muscles, and routine resection of the thoracodorsal neurovascular network including the long thoracic nerve (37, 38).

During the mid-1900s, X-ray diagnosis of breast improved so dramatically that detection of non-palpable tumor in the breast was enabled. This allowed new surgical operations such as lumpectomy, quadrantectomy and segmentectomy to be used.

From 1896, when Beatson published that the surgical castration (bilateral oophorectomy) of two patients resulted in tumor regression, hormonal therapy of breast cancer has progressed through several stages, but correlation between hormones and tumor growth was not proved until hormonal receptors were discovered on breast cancer cells (35). In 1955 Engell published evidence of hematogenous dissemination of malignant cells. This research forced efforts for systemic chemotherapeutic and immunotherapeutic agents for the treatment of metastatic breast cancer.

1.3. EPIDEMIOLOGY

Over one-quarter of the global burden of cancer occurs within Europe despite the fact that Europe's inhabitants comprise only approximately one-eighth of the world's population. The major public health challenges led the European Commission in 1987 to establish a collaborative policy on cancer control. The "Europe Against Cancer Program" identified four separate areas (Eastern, Northern, Southern and Western Europe, figure 1) for action, namely data collection and research, information and health education, early detection and screening, and training and quality control (39).

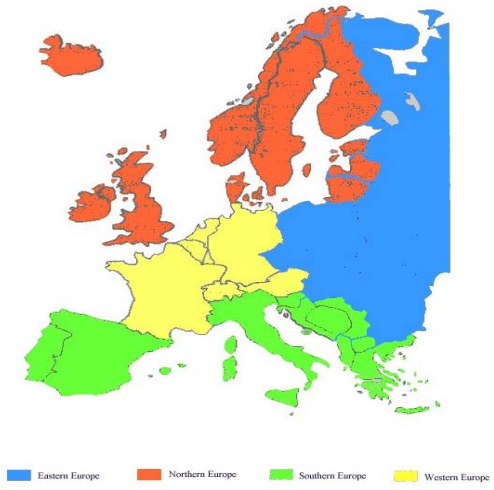

Figure 1. Areas of the "Europe Against Cancer Program" (39).

According to a worldwide study on cancer mortality, Eastern European men had the second highest rates of cancer (414.2), with extremely high rates in Hungary (566.6) and in the Czech Republic (480.5). The rates of cancer in Eastern European women were lower than in the other three areas, although as with men, female rates were very high in Hungary (357.2) and in the Czech Republic (333.6). Generally, mortality rates were highest in Eastern European countries, notably in Hungary, reflecting a combination of poorer cancer survival rates and a higher incidence of the more lethal neoplasms (39, 40 figure 2).

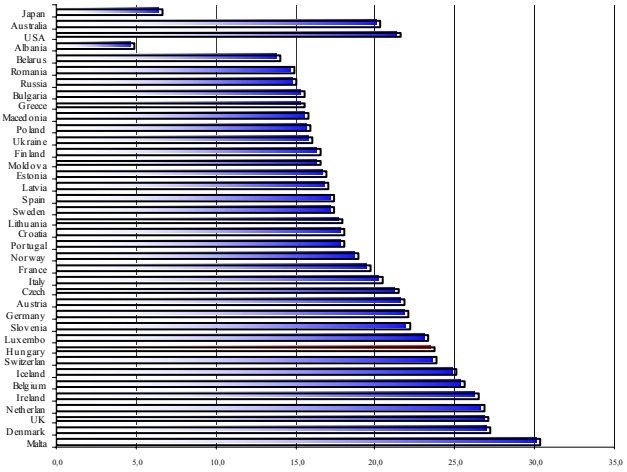

Figure 2. Cancer mortality rates in 1994 (40)

By far the most common cancer in women in Europe is breast cancer. There were an estimated 321.000 new cases in 1995, representing over a quarter of all new cancers occurring in females. The most common primary tumor sites of women are breast (26%), colon and rectum (14%) and stomach (7%). There were approximately 124.000 deaths from breast cancer (17% of all female cancer deaths), and hence it was also the most common cause of cancer mortality in women (53). There are clear geographical differences in risk (figure 3) with high rates of incidence observed in Western Europe. It is likely that the different prevalence of the known risk factors for breast cancer between social classes explain much of the variation, while some of the excess incidence may be attributable to mammographic screening (39).

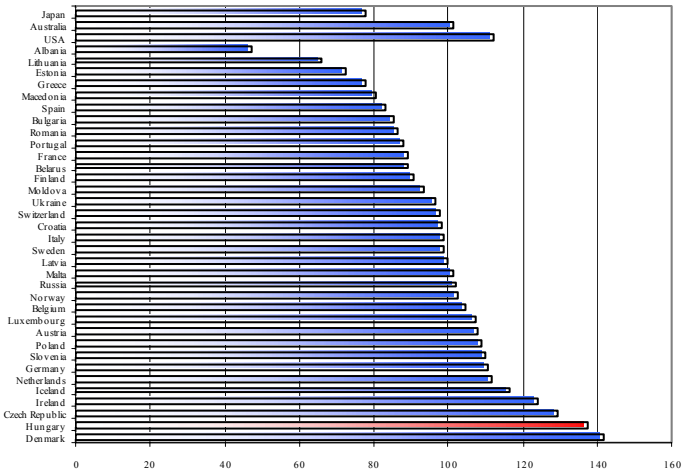

Figure 3. Breast cancer mortality rates in 1994 (54)

According to the data of most countries of the world, except Asia, the incidence and mortality increases with age with the highest rate being among women over 85 where the incidence is over 350/100.000 (41). The risk of breast cancer development in women is one out of eight, which means that every eighth woman in the life period will have breast cancer. Breast cancer occurs 100 times more often in women than in men (42).

Data from 1990 in the USA shows that in every 15th minute 4.28 new cases of breast cancer are registered and within the same period of time one woman dies from breast cancer. Black women have significantly lower incidence of breast cancer (maybe because of earlier and numerous deliveries), but higher mortality, probably due to the later detection, rarely positive hormonal receptors and worse socio-economical status, although molecular factors are not significantly different (43, 44).

Considering the location in the breast, cancer occurs more often in the outer upper quadrant (38.5%) (figure 4) (35).

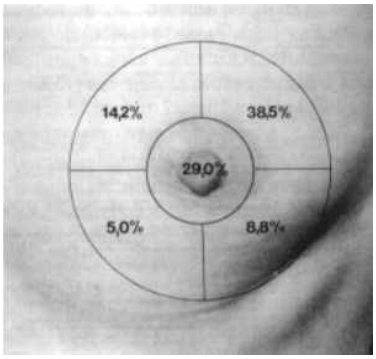

Figure 4. Breast cancer frequency according to the location (35)

In Hungary in 1996, leading death cause of women aged 20-44 and 45-64 on second place behind deaths from cardiovascular diseases was cancerous diseases with 32.7% and 37.7% respectively (figure 5). Differently from the countries of the European Union, cancer mortality shows a continuous increasing tendency with no temporary decreases (45).

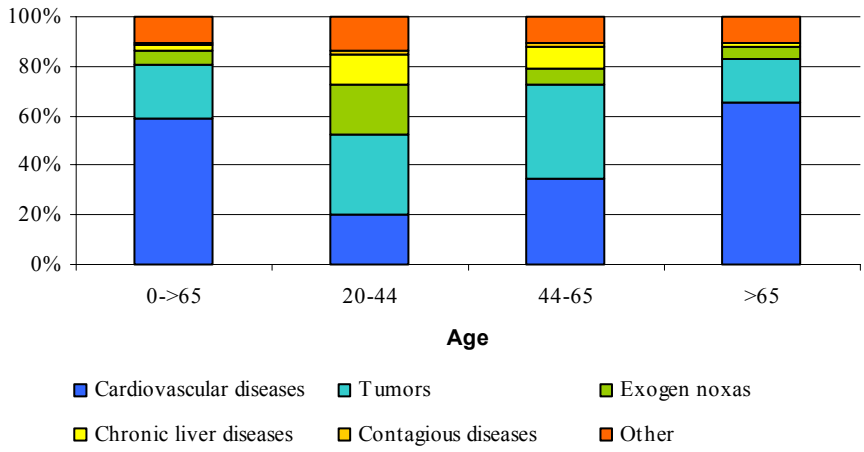

Figure 5. Mortality structure of women in Hungary, 1995 (45)

At the beginning of the 70s, breast cancer incidence and mortality in Hungary was lower than the matching data from the countries of the European Union. Since then, statistics show a declining tendency, and the difference between Hungary and the European Union has already disappeared by the beginning of the 80s. Because of the efforts executed in the European Union, mortality has started to decrease in these countries, showing a continuous decreasing tendency even nowadays (figure 6) (46). In Hungary, continuous increase was observed in breast cancer mortality until the middle of the 90s. Since then, stagnation can be demonstrated (figure 7). According to a study executed in 2003, leading cause of cancerous death of Hungarian women is lung cancer, due to the increasing number of female smokers, not due to the decrease in breast cancer mortality (47).

Prognosis of fully developed breast cancer is not among the worst ones and moreover, it's relatively sensitive to different treatments. Indicators of survival show a continuous increasing in the last two decades (46), though Hungarian practice of mammography is far from considering the frequency of application. Also serious differences exist within the country,

which should be solved to make a progress in breast cancer screening with mammography, early diagnosis and prognosis (46).

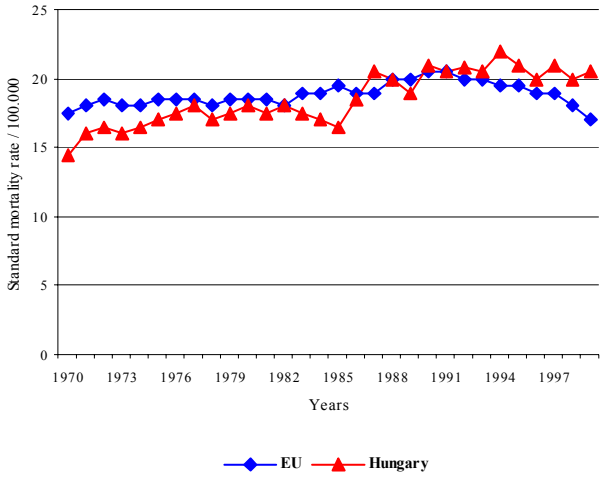

Figure 6. Breast cancer mortality of women (aged 0-64 years) in Hungary and in the European Union, 1970-1999 (56)

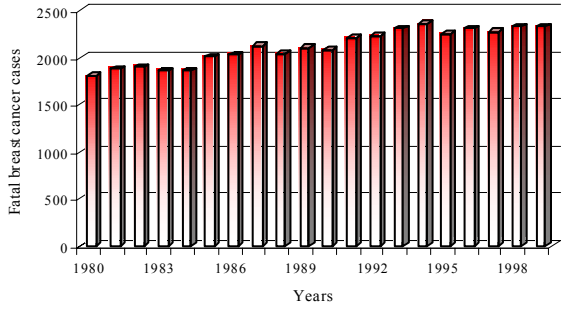

Figure 7. Fatal breast cancer cases in Hungary, 1970-1999 (56)

A national screening program of 10 years was initiated in 2001 in order to increse the efficiency of early diagnosis and to decrease the mortality of breast cancer. The screening rates of women aged 45-65 for 2001 and 2002 were 7% and 21.7%, respectively (figure 8) (48). According to an economics study, in the age group 45-65 with 10% mortality decline 509 lives (“Hungarian trend”), with 20 mortality decline 1074 (“English trend”), while with

30% mortality decline 1582 lives ("Swedish trend") could be saved during a 10 years screening programme (48). Despite the increasing trend of Hungarian women aged 45-65 participating regulary the mammographic screening programmes (figure 8), only about 40% of them is enrolled in the screening, which is still far below the participating frequency of 80% observed in Sweden (49), where a breast screening programme has successfully decreased the breast cancer mortality rate by 30% (48). Considering the above data, rate of Hungarian women aged 45-65 participating regular mammographic screening must be increased in order to decrease breast cancer mortality rate also on Hungary (48).

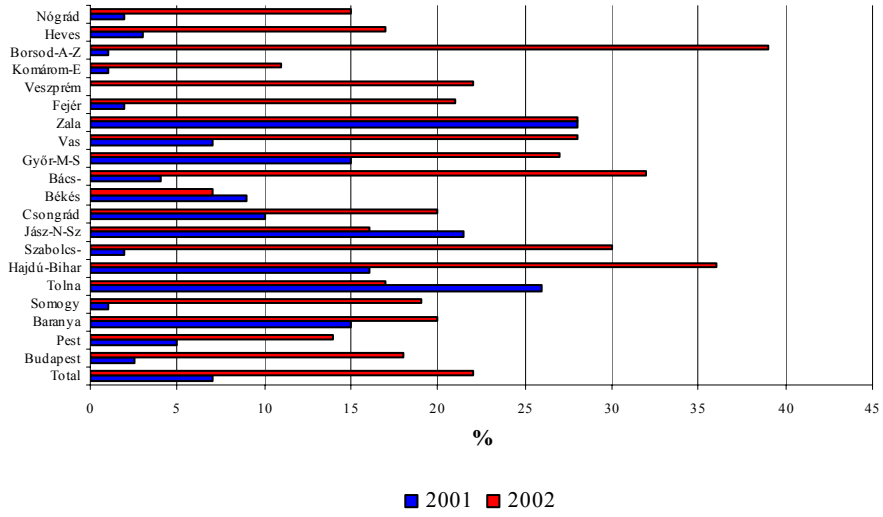

Figure 8. Estimated screening values of mammographic screening among women aged 45-65 in Hungary, 2001-2002 (48)

1.4. ETIOLOGY

Etiological factors responsible for breast cancer development are still not completely known, but epidemiological evidences significantly suggest on three possible groups of genetic, endocrine and exogenous factors (50).

Genetic mutations responsible for breast carcinogenesis are:

1. activation of proto-oncogene (HER2/neu, located at 17q),
2. inactivation (loss or mutation) of tumor suppressor genes: 1p, 1q, 3p, 5p, 6q, 7q, 8p, 9q, 13q, 15q, 16q, 17p (p53), 17q (BRCA1 and NF1) and 18q
3. inactivation of genes responsible for DNA repair (51-62).

Breast cancer family history is important for the first generation of female family members i.e. mother, daughter and sister. Women whose mothers had bilateral breast cancer before menopause carry the highest risk. They have nine times higher risk than others, i.e. 50% of them may develop breast malignancy (50).

Endocrine factors are connected to the endogenous hyperestrogenism, and exogenous intake is connected to the intake of oral contraceptives (OC) and to hormone replacement therapy (HRT). The most important risk factors are: long period of generative time (earlier menstruation and later menopause), infertility, late age at first full-term pregnancy and obesity (63-66).

Influence of physical exercise on the age of the first menstruation is very significant, e.g. girls who exercise regularly whether they practice ballet, swimming or running, start their period later than others. It was demonstrated that girls who did ballet started their menstruation at the average age of 15.4 years in comparison to the control group who started menstruation at the average age of 12.5 years (67).

There are evidences that hyperestrogenism is connected to fibrocystic epithelial hyperplasia. Moderately increased (although disputed) risk is determined by exogenous estrogen (long usage of OC or HRT in menopause). Many studies have been published about the influence of OC and HRT on breast cancer, with controversial results and the only clear conclusion is that they have no protective effect against breast cancer (68-78). Breast cancer cells in women produce different growth factors (TGFα, PDGF). Estrogens stimulate the production of these growth factors and it is possible that interactions of circulating hormones, hormonal receptors of cancer cells and autocrine growth factors have a role in the progression of breast cancer.

Measurement of the quantity of hormonal receptors in breast tissue is used to predict response of breast cancer cells to hormonal therapy (36). In the postmenopausal period larger source of estrogen is fat tissue, where conversion of adrenal androstendion into estrogen occurs (79). Women younger than 50 have little or no increased risk connected to the body mass (BM), while women over 60 with 10 kg overweight have an elevated risk (80%) of breast cancer development (80). Visceral obesity is common in over-weighted patients with breast cancer (81, 65, 79-83).

Exogenous factors are connected to viral infections, higher consumption of alcohol, exposure to ionized radiation (natural and artificial), smoking, long term hair dying and stress (50, 44, 84, 85).

Virus, as an etiological factor of breast cancer, was incriminated in 1936 by Bittner's discovery that a filterable agent, transmitted through mother's milk, causes breast cancer in

suckling mice. The virus, called mouse mammary tumor virus (MMTV), was later recognized as a retrovirus. There are some indications of the existence of a similar virus in human breast cancer, but research results are not convincible yet (44, 86).

According to the numerous prospective studies, alcohol consuming increases the risk of breast cancer development (84).

Selenium level in the sera of the patients with breast cancer is significantly lower than in healthy population (87). Consuming vitamin A, vegetables and fruits in an increased quantity decreases the risk (88). In the Mediterranean countries, the incidence is lower in women who regularly consume olive oil (89).

Radiation exposure increases the risk of breast cancer development. For example, the hydrogen bomb thrown on Hiroshima and Nagasaki significantly increased incidence of breast cancer in that region after a latent period of 20 years. In other words, the highest incidence was noticed in women who were 10 to 14 years old in the moment of explosion and cancer was diagnosed most often when they were 30 to 49 years old (90).

Certain number of patients connects trauma to the disease, but trauma could not be considered a possible risk of breast cancer development. It is quite possible that trauma is just a warning on already existing tumor (90).

It is well known that the incidence of breast cancer in Japan or in China is 4 to 7 times lower than in the USA, but after few generations cancer incidence in Japanese and Chinese immigrants in the USA has become equal to that of the domicile population (91). Thus, international mortality in the period of 1983-1987 varied from less than 6 in Japan to almost 30 in England and Wales (76).

Psychiatric patients have 3.5 times higher incidence of breast cancer in comparison to other patients, i.e. 9.5 times higher in comparison to all the female population. It is not known yet whether stress caused by disease, medication therapy or something else is responsible for the increased risk seen in these patients (92). One of the exogenous predisposed factors in disease development is stress, however, as we find stress a hard subject to any kind of measurement, there are little information about it in the literature.

On the other hand, there are a few cases of breast cancer where none of the aforementioned factors could be identified.

1.5. PATHOLOGY

The breast is composed of epithelium, connective and fat tissue, so tumors developed in the breast are the tumors of these tissues, and can be benign or malignant. Most of the malignant breast tumors originate from the epithelium (41).

There are several classifications of malignant breast tumors but two of them are used the most: classification according to the World Health Organization (WHO) (table 1) and classification according to the Armed Forces Institute of Pathology (AFIP) (table 2) (93, 94).

Regarding the relationship of malignant cells to basal membrane, cancers can be noninvasive (cells do not invade the basal membrane) or invasive (cells do invade the basal membrane). The most common pathohistological type of breast cancer is the invasive ductal carcinoma (IDC) (>80% of all breast cancers) followed by the invasive lobular carcinoma (ILC) (10%) and medullary carcinoma (5%) which is less common at older age. Mucinous and papillary carcinomas are more common among older women, but make less than 10% of all breast cancers (41).

A. Noninvasive	B. Invasive (infiltrating)
1. Intraductal carcinoma	1. Invasive ductal carcinoma - not otherwise specified (IDC)
2. Intraductal papillary carcinoma	2. Invasive lobular carcinoma
3. Lobular carcinoma in situ	3. Medullary carcinoma
	4. Colloid carcinoma (mucinous carcinoma)
	5. Paget's disease
	6. Tubular carcinoma
	7. Adenoid cystic carcinoma
	8. Invasive comedo carcinoma
	9. Apocrine carcinoma
	10. Invasive papillary carcinoma

Table 1. WHO classification of breast cancer (95)

Epithelial cancers	
Non-invasive	Intraductal carcinoma
	Intraductal carcinoma with Paget's disease
	Lobular carcinoma in situ
Invasive	Invasive ductal carcinoma
	Invasive ductal carcinoma with Paget's disease
	Invasive ductal carcinoma with predominant intraductal component
	Invasive lobular carcinoma
	Medullary carcinoma
	Mucinous carcinoma
	Invasive papillary carcinoma
	Tubular carcinoma
	Adenoid cystic carcinoma
	Secretory (juvenile) carcinoma
	Apocrine carcinoma
	Carcinoma with metaplasia
	Carcinoma with giant cells that are like osteoclasts
	Cystic hypersecretory carcinoma with invasion
	Carcinoma with endocrine differentiation
	Carcinoma rich with glycogen
	Carcinoma rich with lipids
	Invasive cribriform carcinoma
Clinical types of cancer	Inflammatory breast carcinoma
	Carcinoma during the pregnancy and lactation
	Occult carcinoma with metastases in the axillary lymphatic nodules
	Carcinoma of the ectopic breast
	Carcinoma in men
	Carcinoma in children
Mixed cancers of the epithelium and of the connective tissue	Malignant cystosarcoma phylloides
Mesenchymal cancers	Angiosarcoma
	Fibrosarcoma
	Leiomyosarcoma
	Chondrosarcoma
	Osteosarcoma
	Haemangioperycitoma
	Dermatofibrosarcoma protuberans
Cancers of the breast skin	Melanoma of the nipple
	Carcinoma of the squamous cells of the nipple
	Carcinoma of the basal cells of the nipple
	Carcinoma of the skin
Cancers of the lymphatic and hemopoetic tissue	Non-Hodgkin lymphoma
	Plasmacytoma
	Leukemical infiltration
	Hodgkin disease

Table 2. AFIP classification of malignant breast tumors modified according to Rosen (93, 94).

Histopathological grading of breast cancer according to Blom-Richardson is also important to mention. It shows the way of growth of IDC and the cytological characteristics of differentiation (table 3) (95, 96).

Grading parameters	
a) Formation of the gland tubules and acini	1 point: characteristic formation of tubules (>75%) 2 points: moderate formation of tubules (10-75%) 3 points: little or without tubules at all (<10%)
b) Pleiomorphism of cancer cells nucleus (abnormality in size, shape and structure)	1 point: isomorphism of nuclei 2 points: moderate variability in size, shape and in structure of nucleus 3 points: characteristic polymorphism
c) Mitoses / 10 hpf	1 point; < 9 mitosis 2 points; 10-19 mitosis 3 points; > 20 mitosis

After summing the points of all parameters the level of differentiation can be determined according to the following scheme:

G1 (3-5 points)	well differentiated
G2 (6-7 points)	moderately differentiated
G3 (8-9 points)	poorly differentiated

Table 3. Histopathological grading of IDC according to Blom-Richardson – Elston's modification (95, 96)

Ductal carcinoma is the most common and most aggressive form of breast cancer. According to certain authors it makes 90% of all beforehand-undiscovered cases found by mammography. It becomes invasive very often, usually in half the time period than lobular: it takes 20 years for the intralobular form to become invasive, while for ductal carcinoma it takes only 10 years. Ductal cancers are divided into subtypes: comedocarcinoma, cribriform, apocrine, papillary, micropapillary and solid type. Comedo and cribriform types are the most aggressive ones (41).

Lobular carcinoma represents 11% of all breast cancers. Its characteristic is the bilateral manifestation, whether at the same time or in one breast after another.

IDC is the most common type of breast cancer (>80% of all breast carcinomas). The cancer is obviously invasive even macroscopically and invades connective tissue stroma. Small foci of calcifications are often evident on the cut surface. It could cause retraction of the skin and/or the nipple and fixation to the underlying chest wall. Histologically, connective stroma can be seen with focuses or rays of tumor cells scattered about. At the edges of the tumor, malignant cells infiltrate surrounding tissue, very often invading perivascular and perineural spaces as well as lymphatic and blood vessels (figure 9) (97).

Figure 9. Invasive ductal carcinoma of the breast (97)

Tubular carcinoma or well-differentiated adenocarcinoma is diagnosed in 10 to 20% of the breast cancer cases and it can be relatively easily discovered by mammography. Macroscopically, tubular cancer is usually a small lesion, often smaller than 1 cm. Histologically, areas of sclerosis or deposits of elastin can be seen. Tubular cancer is often combined with intraductal cancer (65% of the cases). Tumor cells are often atypical and show numerous mitoses. About 10% of tubular cancers are metastatic. (figure 10) (41).

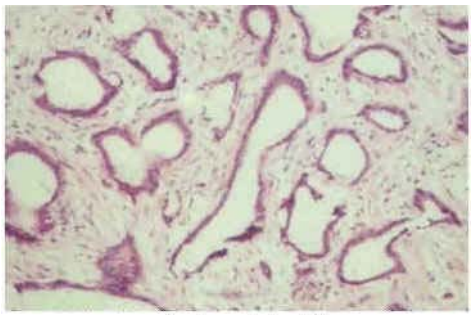

Figure 10. Tubular carcinoma of the breast (97)

Lobular carcinoma is developed from one or more terminal ducts and/or from ductules (acini). It often appears bilaterally (20%). Two types are described: lobular cancer in situ and intralobular carcinoma. In the first type cells are bigger than normal, with oval or round nuclei and small nucleoli, neither mitoses nor polymorphism can usually be found. Dilatation of the acinus is characteristic for intralobular carcinoma. About 30% of patients develop a second malignancy in the same or in the other breast, and the developed infiltrating carcinomas can be either lobular or ductal. Intralobular cancer makes 5 to 10% of all the breast carcinoma cases. Macroscopically, lobular carcinoma is poorly edged and usually of rubber consistency, sometimes hard and scirrhous. Histologically, cancer cells are mostly small and uniformed with small rate of polymorphism (figure 11) (41)

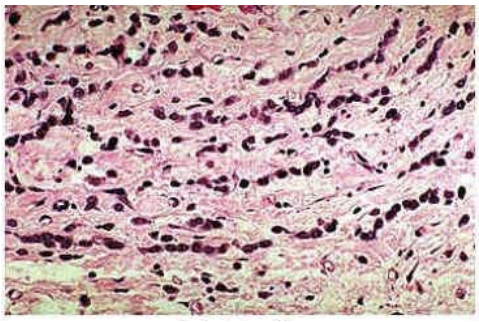

Figure 11. Invasive lobular carcinoma of the breast (97)

Medullary carcinoma represents about 1% of all the breast cancers. It is rather soft and fleshy than hard on external palpation. Histologically, the carcinoma is characterized by solid, syncytium-like sheet of large cells that are mainly undifferentiated, although sometimes are well differentiated. Lymphatic infiltration is a common finding (figure 12) (41).

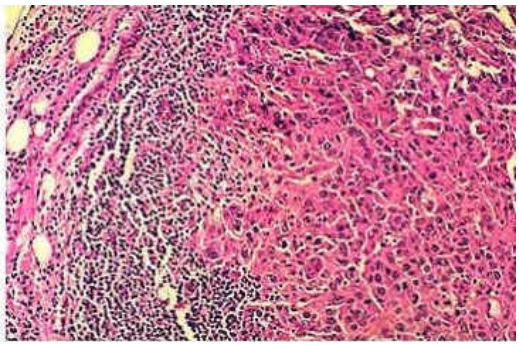

Figure 12. Medullary carcinoma of the breast (97)

Colloid or mucinous carcinoma (carcinoma colloides seu mucinosum) is characterized by intracellular and extracellular mucinous formation. Macroscopically, colloid carcinoma is consisted of tender and extensive gray-blue nodules of gelatinous consistency. Histologically, there are two forms of tumor: 1) cancer cells are visible as small islands or even as isolated cells that float in great lakes of mucine, which leaks into the surrounding tissue space, 2) cells grow in well-presented gland formations. In both forms, tumor cells can be vacuolated by the mucine content (figure 13) (41).

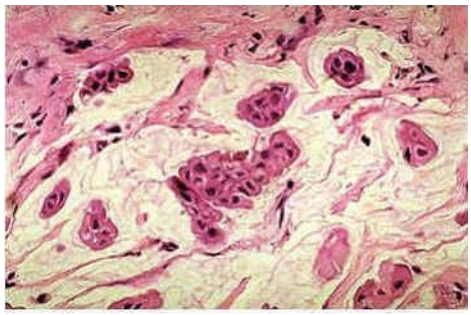

Figure 13. Colloid/mucinous carcinoma of the breast (97)

Paget's disease (morbus Paget) is a special type of ductal carcinoma, affecting women of older age. Development of the disease starts as typical intraductal cancer that arises from the main excretory ducts of the breast and extends intraepithelially to involve the skin of the nipple and the areola. The affected skin is frequently fissured, ulcerated and oozing. Surrounding inflammatory hyperemia, edema and also bacterial infections are often found. The histologic hallmark of this tumor is invasion of the epidermis with characteristic tumor cells called Paget's cells. These cells are large and hyperchromatic, surrounded with a lightly stained ring that represents intracellular deposit of mucopolysaccharides. The morphologic picture is similar to that of the intraductal carcinoma, but this type of cancer has better prognosis (figure 14) (41).

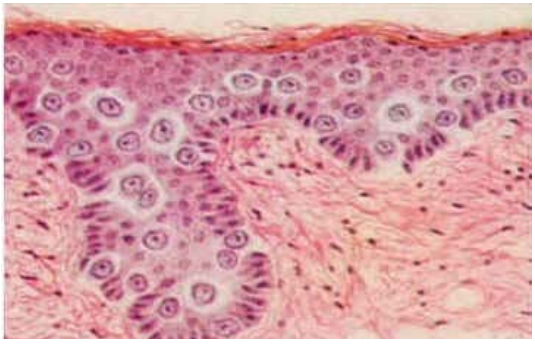

Figure 14. Paget's disease (97)

1.6. CLINICAL DIAGNOSIS

Main diagnostic methods for detection of breast cancer are anamnesis, physical examination and mammography. However, a biopsy for pathological evaluation should be performed for exact diagnosis in every case. Different techniques are used to obtain pathological material, but two of them are mostly used: 1) cytological evaluation of the breast discharge i.e. of the aspirated material from the breast, 2) intra-operative biopsy with histological evaluation of the frozen section. The first method is suitable for diagnosis, the second one is extremely valuable in selecting treatment for breast cancer depending on histological verification of the relationship of malignant cells towards basal membrane, in other words, whether the cancer is invasive or non-invasive.

Indirect diagnostic methods are self-examination (harmless, easy to learn, free of charge), anamnesis, physical examination, native mammography, ultrasound and new diagnostic techniques such as MRI (Magnetic Resonance Imaging), digital mammography, CAD (computer-aided diagnosis), PET (positron emission tomography), SPECT (single photon emission imaging and computed tomography), thermography, diaphanoscopy and use of molecular markers (98).

Anamnesis is consisted of two parts:

- *Family history:* informs about any illness and causes of death in the family of the patient, with emphasis on the breast diseases of female relatives (grandmother, mother, aunts and sisters of patient).
- *Personal history:* provides information about the age of menarche, the beginning of sex life, age at first delivery, number of deliveries, age of menopause. The patient should also be asked whether she performs breast self-examination, has noticed any change in her breast, about the number and intervals of previous physical breast examinations, previous diagnostic procedures of the breast, usage of OC and HRT.

Physical breast examination should be done both in sitting and supine positions. A thorough physical breast examination should be done to locate any lump or suspicious area. The skin of the breast and the nipples should also be carefully inspected. It is important to notice any discharge from the nipple. The lymph nodes under the armpit should be palpated too.

Although, by this examination it is possible to discover only lumps greater than 1 cm, its importance is very emphasized, especially when combined with mammography due to its importance as a complementary examination method (98). Sometimes, ultrasound can give dubious results although the result of mammography is negative (99). Sensitivity of mammography, ultrasound and palpation is shown in figure 15 (99).

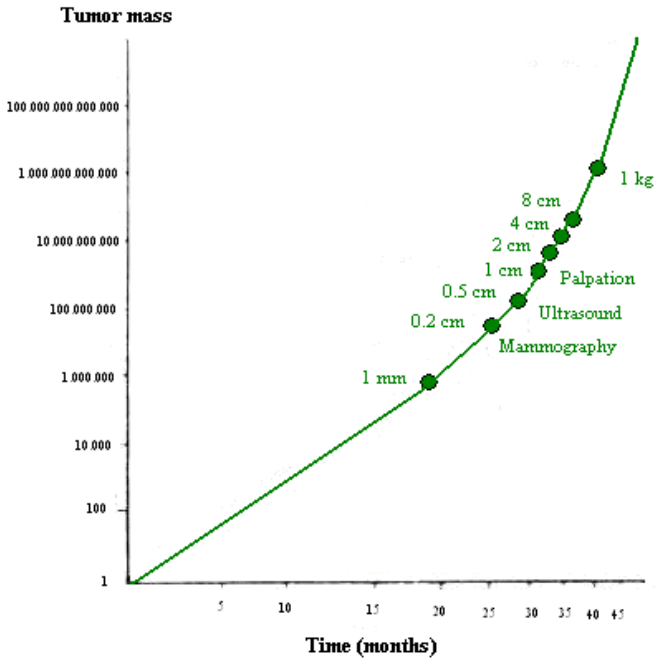

Figure 15. Sensitivity of palpation, ultrasound and mammography in detecting breast cancer (99)

Mammography and ultrasound of breast

Ultrasound (after physical examination) is an important diagnostic technique usually applied in the population of younger women (figure 16), due to its sensitivity and specificity that is higher in this period compared to mammography (figure 17), because the largest part of the breast in the generative age is made of hyperechogen gland tissue, while in the menopause gland tissue is replaced with hypoechogen fat tissue (99).

Figure 16. Ultrasonography – ductal mammary carcinoma

Figure 17. Mammography

In elderlies, methods are complementary, but since mammography is easier to perform and adequately sensitive, it is preferred to ultrasound in this population. In order to decrease the number of unnecessary biopsies, each suspicious change should be verified by cytopunction. Moreover, certain number of breast cancers can not be discovered by mammography, 10-25% of the palpable breast cancers are not visible by mammography, and patients discover an additional 20-25% in the period between mammographic and clinical controls (100).

Molecular markers of breast cancer are normally existing compounds of the body, and are found in higher concentration in patients with breast cancer than in healthy women. The level of the carcino-embryonic antigen (CEA) is increased by a few percentages in stage I and II breast cancers. Though CA15-3 and CA549 markers are elevated in 20% to 50% of patients with primary breast cancer, they can also be found in a higher concentration in 20% of patients with benign breast lesions and with gastrointestinal diseases. Cathepsin D could be more important in diagnostic testing due to its specificity in breast cancer. But none of these markers are specific only for breast cancer, and could even in combination imply to early stage of the breast cancer. In order to propose an appropriate diagnosis of breast cancer and to observe treatment success, elementary laboratory techniques such as sedimentation, total blood exam, biochemical examination (SGOT, SGPT, γGT, ALP with isoenzymes, LDH) are needed. X-ray of lung for detecting possible metastases is compulsory before any therapeutic treatment (101-104).

1.7. CLASSIFICATION

1.7.1. TNM CLASSIFICATION

Besides histopathological features, the clinical stage of the tumor is also considered an important factor in determining prognosis and proper treatment. Pierre Denoix developed the basic classification of the malignant tumors according to their dissemination (1943-1953). He took tumor seize (T), tumor dissemination to regional lymph nodes (N), and presence of metastasis (M) into consideration in his classification system, named TNM system. Today,

staging of cancer is determined by the UICC (Union International Contre le Cancer) classification based on the TNM system (table 4). It was changed in 1987 to bring UICC's and AJCC's (American Joining Commission on Cancer Staging and End Results Reporting) classifications closer to each other. The final classification system is a result of clinical, radiological and laboratory investigations (44).

Tx	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ(DCIS: ductal carcinoma in situ, LCIS: lobular carcinoma in situ, Paget's disease of the nipple with no tumor
T1	Tumor 2 cm or less in greatest dimension
T1mic	Microinvasion 0.1 cm or less in greatest dimension
T1a	Tumor more than 0.1 cm but not more than 0.5 cm in greatest dimension
T1b	Tumor more than 0.5 cm but not more than 1 cm in greatest dimension
T1c	Tumor more than 1 cm but not more than 2 cm in greatest dimension
T2	Tumor more than 2 cm but not more than 5 cm in greatest dimension
T3	Tumor more than 5 cm in greatest dimension
T4	Tumor of any size with direct extension to chest wall or skin, only as described below
T4a	Extension to chest wall, not including pectoralis muscle
T4b	Edema (including peau d'orange) or ulceration of the skin of the breast or satellite skin nodules confined to the same breast
T4c	Both T4a and T4b
T4d	Inflammatory carcinoma
Nx	Regional lymph node metastasis cannot be assessed (e.g., previously removed)
N0	No regional lymph node metastasis
N1	Metastasis to movable ipsilateral axillary node(s)
N2	Metastasis to ipsilateral axillary lymph nodes fixed or matted, or in clinically apparent ipsilateral internal mammary nodes in the *absence* of clinically evident axillary lymph node metastasis
N2a	Metastases in ipsilateral axillary lymph nodes fixed to one another (matted) or to other structures
N2b	Metastasis only in clinically apparent ipsilateral internal mammary nodes and in the *absence* of clinically evident axillary lymph node metastasis
N3	Metastasis in ipsilateral infraclavicular lymph node(s) with or without axillary lymph node involvement, or in clinically apparent ipsilateral internal mammary lymph node(s) and in the *presence* of clinically evident axillary lymph node metastasis; or metastasis in ipsilateral supraclavicular lymph node(s) with or withour axillary or internal mammary lymph node involvement
N3a	Metastasis in ipsilateral infraclavicular lymph node(s)
N3b	Metastasis in ipsilateral infraclavicular lymph node(s) with or without axillary lymph node involvement, or in clinically apparent ipsilateral internal mammary lymph nodes the persence of clinically evident axillary lymph node metastasis; no metastasis in ipsilateral supraclavicular lymph nodes with or without axillary or internal mammary lymph node involvement
N3c	Metastasis in ipsilateral supraclavicular lymph node(s)
Mx	Distant metastases cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Table 4. TNM breast cancer classification (44)

1.7.2. STAGING

Staging refers to the grouping of patients according to the extent of their disease. Staging is important to determine treatment for individual patients, estimate their prognosis and compare the results of different treatment programs (table 5 and figure 18) (100, 105).

	T (tumor)	N (nodes)	M (metastases)
Stage 0	Tis	N0	M0
Stage I	T1	N0	M0
Stage IIA	T0	N1	M0
	T1	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	
	T4	N2	
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Table 5. Staging of breast cancer (100, 105)

STAGE GROUPING

Stage 0	Tis	N0	M0
Stage I	T1*	N0	M0
Stage IIA	T0	N1	M0
	T1*	N1	M0
	T2	N0	M0
Stage IIB	T2	N1	M0
	T3	N0	M0
Stage IIIA	T0	N2	M0
	T1*	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
Stage IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
Stage IIIC	Any T	N3	M0
Stage IV	Any T	Any N	M1

Figure 18. Staging of breast cancer

1.8. DIFFERENTIAL DIAGNOSIS

There is an important principle that each suspicious change in the breast must be considered as a malignant tumor until proved differently. This principle imposes biopsy of all the lesions. It is prudent to pay attention to some of the lesions that appear more often at certain age in women. Cystic hyperplasia, fibroadenoma and mastitis occur more often before the age of 35. Most tumors of the breast develop between ages 40 and 60. Incidence of breast cancer is growing with the age.

1.9. TREATMENT

Breast cancer treatment is done according to different protocols depending on the histological type of the tumor, stage of the malignant process and the overall physical condition of the patient. Different combinations of surgical treatment, radiotherapy, chemotherapy, hormonal therapy and immunotherapy are in use. In most cases treatment begins with surgery. Nowadays, radical mastectomies according to Halsted and superradical interventions with thoracotomy are replaced with breast-conserving surgery like quadrantectomy. In patients with histological signs of invasive breast cancer, besides surgery and irradiation, systemic therapy is often used in order to prevent relapses.

1.10. PROGNOSIS

Some of the prognostic factors, like tumor size, histological differentiation are reliably approved, while reliability of others still needs to be approved. According to histological classification, invasive ductal carcinoma is the most malignant lesion, followed by invasive lobular carcinoma. Medullary and mucinous carcinoma usually have better prognosis.

It is known that tumor development, as well as prognosis, depends on histological differentiation. Well-differentiated tumors develop slower and metastize later. Poorly differentiated tumors are more malignant, anaplastic tumors being the worst. An inflammatory cell reaction composed of lymphocytes and/or plasma cells in tumor stroma and around metastases is a good prognostic factor.

The Breast Cancer Detection Demonstration Project study demonstrated that survival rate depends not only on the clinical stage of disease but also on patient's age (figure 19 and 20) (106, 107).

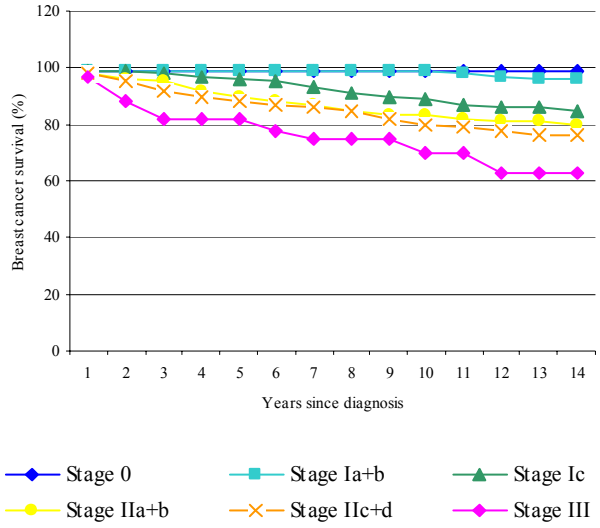

Figure 19. Survival by stage at diagnosis among women aged 40-49 at diagnosis (106)

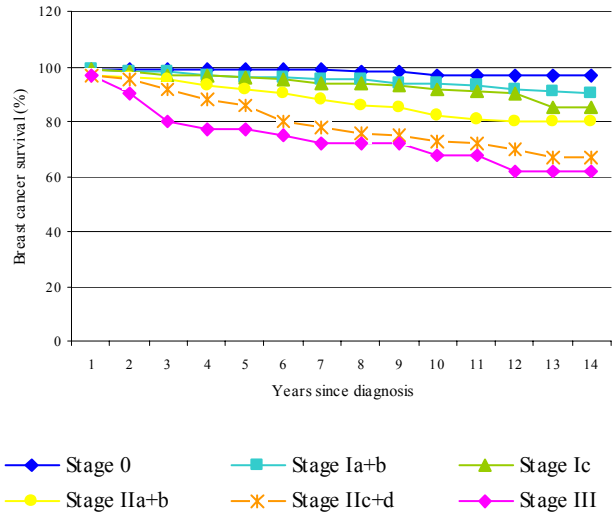

Figure 20. Survival by stage at diagnosis among women aged 50-59 at diagnosis (107)

The 5-year disease free survival (DFS) for patients with localized and properly cured breast cancer is 98%, and 95% after 10 years, whereas patients with metastasis have 30% DFS and 50% after 10 years (107).

Recently, breast cancer research is mainly related to molecular biology. Some of the new insights are shown below:

Patient age (35, 44, 50, 85, 107)

Obesity (35, 44, 66, 80)

Relationship of tumor and basal membrane (35)

Size of the primary tumor (35, 44)

Histological type of tumor (35, 41, 44)

Histological differentiation of tumor (35, 44, 105)

Proliferating ability of tumor cells (mitotic index, index of cells labeled with 3H thymidine-LI) (35, 44)

Characteristics of the inflammatory reactions in the tumor (35, 44)

Presence of blood vessels invasion (35, 44)

Status of lymph nodes (number, localization and size of positive lymph nodes) (35, 44, 103)

Metastases and their locations (35, 44)

Expression of the estrogen and progesterone receptors (64)

Ploidy / DNA index (56, 71)

Proliferating cell nuclear antigen / PCNA (35)

Proliferation marker MB-1 (35)

Expression of HER2/neu oncogene (44)

Expression of tumor suppressor gene p53 (3, 4, 5)

Expression of tumor suppressor gene MMP2 (35)

Expression of tumor suppressor gene nm23 (44)

Expression of receptors for EGFR (145, 146)

Expression of laminine receptors (LR) (35)

Srp-27 protein expression (57)

Cathepsin D expression (57)

Level of the 5-hydroxymetil-2'-deoxyuridin UDNA (57)

1.11. SCREENING

Because of the connection between early detection and improved outcome, proper screening method is the main goal in breast cancer cases. Many studies have demonstrated that screening of breast cancer is evidently useful for women aged 50 to 74, but it is under debate for women aged 49 and younger (108-110). Methods of early detection are self-examination, physical examination, ultrasound and mammography (38).

The best guidelines for early detection of breast cancer are shown in figure 18. Women aged 20 or older should perform breast self-examination every month. Over age 35, women should have a breast examination performed by health professionals every year, women should have baseline mammography at age 40 or even earlier if they are in a high-risk category (after age 35). Mammography should be repeated every two years and every year for women aged 50 (110). Regular mammograms (figure 21) can decrease breast cancer mortality of women aged 50 to 69 years by 30% (110).

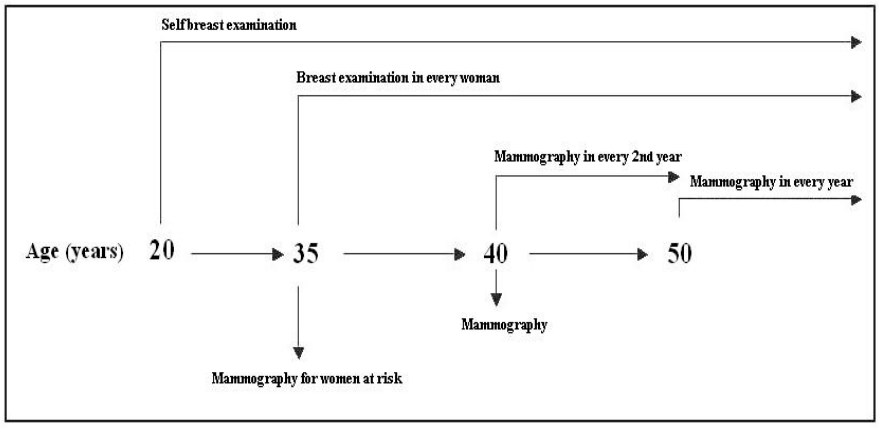

Figure 21. Guidelines for early detection of breast cancer

2. MATERIALS AND METHODS

2.1. Tissue samples

Seventy-two BC specimens, 72 autologous PTT sampled at 1 cm, 65 at 2 cm and 43 at 3 cm from the primary BC and 44 mammary BL were prospectively collected for immunohistochemical analysis (IHC) and fluorescent in situ hybridization (FISH) from patients surgically treated at Regina Elena Cancer Institute.

Further hundred and eighty-six patients receiving anthracycline-based adjuvant therapy were selected for studying the COX2, hormone receptors, HER2, p53, Ki67, Fas, FasL, PGE_2S and EGFR expression in primary breast cancers and in the their autologous lymph node metastases.

2.2. Immunohistochemistry

IHC staining was carried out on 5 μm thick sections on silane (APES, Sigma, St. Louis, MO, USA) treated slides for routinely fixed paraffin embedded blocks. The

deparaffinized and rehydrated sections were pretreated twice in microwave oven at 750 W for 5 min in citrate buffer pH=6 and incubated for 60 min. at room temperature with primary antibodies. The reaction was visualized using a streptavidin-biotin immunoperoxidase system (LSAB2 kit, DakoCytomaton, Milan, Italy) and 3-amino-9-ethyl-carbazole solution (DakoCytomaton) as chromogenic substrate. Sections were then slightly counterstained with Mayer's haematoxylin and mounted in aqueous mounting medium (Glycergel, Dakocytomaton).

Antibodies and working solutions used for immunohistochemistry are shown in table 6. Overexpression of HER2 oncogene product was determined using the high affinity monoclonal antibody (mAb) 300G9 recognizing an epitope of the $gp185^{erbB-2}$ extracellular domain not cross-reacting with EGFR (111) and mAb CB11, which recognizes a peptide of the intracellular domain of the receptor (BioGenex, Menarini, Italy). To verify the benign nature of 1 cm PTT, smooth muscle actin staining was performed with using monoclonal mouse anti-human anti-actin (smooth muscle) antibody 1A4 (DakoCytomation). p53 protein expression was evaluated using the murine mAb DO7 (DakoCytomation). Fas protein was detected by using a commercial mAb (Novocastra) raised against a peptide corresponding to amino acids 316-335, mapping at the carboxy terminus of human Fas. FasL expression was evaluated using N-20 mAb (Novocastra), which recognizes epitopes corresponding to amino acids 2-19 mapping at the amino terminus of the human protein. Estrogen (ER) and progesterone (PgR) receptors were assayed with using commercially available antibodies (ER1D5 and 1A6 Immunotech, UCS, Rome, Italy). To assess the proliferative activity of the tumors, immunostaining with monoclonal antibody Mib-1 (DakoCytomation) was performed. For COX2 investigations antigen was retrieved using microwave (two cycles of 750W, 5 min in citrate buffer) and sections were incubated for 60 min at room temperature with COX2-specific human polyclonal primary antibody (125 ng/µl, 160107, Cayman Chemical Co., Ann Arbor, MI, USA). The reaction was visualized using a streptavidin-biotin immunoperoxidase and chromogenic substrate system (Histostain-Plus, Broad Spectrum (DAB), 85-9643, ZYMED Laboratories Inc., CA, USA). PGE_2S immunohistochemistry was performed with a PGE_2S-specific rabbit polyclonal primary antibody (125 ng/µl, 160140, Cayman Chemical Co., Ann Arbor, MI, USA) while EGFR activity was examined with a commercial mouse primary antibody (750 ng/µl, 28-0005, ZYMED Laboratories Inc., CA, USA). In case of EGFR antigen was retrieved using pepsine (Digest-All 3 ready-to-use pepsine solution, 00-3009, ZYMED Laboratories Inc., CA, USA) for 10 min. at 37°C, otherwise the reactions were performed both for PGE_2S and EGFR as described for COX2 immunoreaction.

Antibody	Working solution	Origin
HER2 300G9	1:200	BioGenex, Menarini, Italy
HER2 CB11	1:200	BioGenex, Menarini, Italy
Actin 1A4	1:300	DakoCytomaton, Milan, Italy
p53 D07	1.300	DakoCytomaton, Milan, Italy
Fas (CD95)	1:50	Novocastra Laboratories Ltd., Milan, Italy
FasL	1:50	Novocastra Laboratories Ltd., Milan, Italy
ER ER1D5	1:300	Immunotech, UCS, Rome, Italy
PgR 1A6	1:300	Immunotech, UCS, Rome, Italy
Mib-1	1:300	DakoCytomaton, Milan, Italy
COX2	1:200	Cayman Chemical Co., Ann Arbor, MI, USA
PGE_2S	1:200	Cayman Chemical Co., Ann Arbor, MI, USA
EGFR	1:200	ZYMED Laboratories Inc., CA, USA

Table 6. Antibodies used for immunohistochemistry

Immunostained slides were analyzed and scored independently by 2 investigators. For HER2 staining in tumors the score was defined as 3+/2+ when more than 10% of the neoplastic cells displayed strong plasmamembrane immunoreactivity, 1+ when <10% of the cells displayed weak positive reaction and negative when no stain was observed.

In the case of p53, immunoreactivity was considered positive when a distinct nuclear stain was observed in at least 10% of the tumor cell population. Since patterns of p53 reactivity in benign lesions and PTT samples may differ from those in breast cancers, the case was scored positive even if scattered nuclei were positive.

As in regard of Fas and FasL, benign and malignant tissues displayed the following two patterns of immunoreactivity: 1) a cell membrane staining associated with a granular cytoplasmic reactivity, and 2) a diffuse cytoplasmic staining of variable intensity. Specimens with a faint, and/or questionable cytoplasmic staining were scored negative. Immunostaining was scored as follows: negative (no expression), heterogenous (expression in 10-50% of the cells) and homogenous (expression in >50% of the cells). Fas and FasL staining patterns in benign lesions and PTT samples were not different from those observed in tumor samples, therefore the same scoring criteria were used.

The following criteria were agreed upon for COX2 before the analysis: 0: no staining, 1+: weak diffuse cytoplasmic staining (may contain stronger intensity in less than 10% of the malignant cells), 2+: moderate to strong granular cytoplasmic staining in 10-90% of the cancer cells, 3+: over 90% of the tumor cells stained with strong intensity (112). For further analysis COX2 immunostaining in the investigated cases was considered only positive or negative.

Regarding Ki67 staining, for each section, $5x10^2$ tumor cells in 4 random fields were counted to determine the percentage of Ki67 positive nuclei. Ki67 index was classified high when >30% (median value).

ER, PgR, PGE_2S and EGFR staining was each considered positive when ≥10% of the cells showed immunoreactivity. In case of less or no positive cells reaction was considered negative.

2.3. FISH

PathVysion HER2 DNA Probe Kit (Abbott Diagnostici, Rome, Italy) was used to determine the HER2 DNA amplification in breast cancer and PTT samples. The LSI HER2 (Spectrum Orange) probe (Abbott Diagnostici, Rome, Italy) contains DNA sequences specific for the HER2 human gene locus and hybridizes to 17q11.2-q12 region of human chromosome 17. The CEP17 (chromosome enumeration probe, Spectrum Green) probe (Abbott Diagnostici, Rome, Italy) contains alpha-satellite DNA that hybridizes to the D17Z1 locus. These two probes were premixed and pre-denatured. CEP17 was used as a control to determine copy number of chromosome 17 in order to adjust for the effect of aneuploid chromosome 17 when the HER2 copy numbers were counted. The ratios of average copy numbers per cell were calculated to establish the presence of amplified HER2. After pretreatment, the procedure “Hybrite System” (Vysis, Abbott Diagnostici, Rome, Italy) was used. Hybrite is an open system for hands-free denaturation and hybridization when using in situ DNA probe procedure. After overnight hybridization at 37 °C, post-hybridization wash was applied using 2xSSC/NP40 (Abbott Diagnostici, Rome, Italy) at 73°C. Tissue sections were counterstained with DAPI (4,6-diamidino-2-phenylindole, Abbott Diagnostici, Rome, Italy). The slides were processed with Olympus BX60 fluorescence microscope (Olympus Italia, Segrati, Italy) equipped with a 100-watt mercury lamp. Separate band pass filters were used for the detection of the HER2 probe signals (Spectrum Orange), CEP 17 probe signals (Spectrum Green) and DAPI counter stain. Fluorochrome signals were captured individually and images were generated via computer with Quips Genetic Workstations and Imaging

Software (Vysis, Abbott Diagnostici, Rome, Italy). The slides were observed at 1000x magnification. At least 100 well-defined nuclei were scored for each hybridization processes. Clumps, overlapping nuclei and tumor infiltrating leucocytes were disregarded. Only nuclei with unambiguous chromosome 17 centromeric hybridization signals were scored for the HER2 signal numbers. The amplification was defined as a HER2 to CEP 17 ratio >2.

2.4. Statistical analysis

Association between clinical and biopathological variables were evaluated using the chi-square test. All these parameters were treated as dichotomous or categorical variables and described using the Pearson statistics. The disease-free (DFS) and overall survival (OS) curves were estimated by the Kaplan-Meier product-limit method. Log-rank test was used to assess differences between subgroups. Significance was defined at the $p<0.05$ level. The relative risk and the confidence limits were estimated for each variable using the Cox univariate model and adopting the most suitable prognostic category as reference group. A multivariate Cox proportional hazard model was also developed using stepwise regression (backward selection) with predictive variables which were significant in the univariate analyses. Enter limit and remove limit were $p=0.10$ and $p=0.15$ respectively. All analyses were conducted using the BMDP software package (Chicago, IL).

3. RESULTS

3.1. Morphological features of peritumoral tissues and benign breast lesions

The 72 breast cancer patients selected for the PTT studies had a mean age of 51.6 years (range 36-88), including pre- and postmenopausal women. Of the 72 breast carcinomas, according to AFIP classification, 62 were diagnosed as infiltrating ductal carcinomas and 10 as infiltrating lobular carcinomas. Tumor sizes varied from T_1 to T_3, (T_1 in 39 cases, T_2 in 31 cases and T_3 in 2 cases). Forty-one of the patients were node negative while 31 tumors had associated nodal metastases. Estrogen receptor positivity was found in 48 cases and progesterone receptors were detected in 49 tumors. Nineteen malignant lesions overexpressed HER2 and 32 proved to be p53 positive. Fas positivity occurred in 31 cases whereas 39 carcinomas showed strong FasL reactivity (table 7).

The histopathological features of the 72 PTT sampled at 1, 2 and 3 cm from the autologous BC to determine HER2, p53, Fas and FasL expression are summarized in table 8. Fibrocystic

changes (FC) were variably associated to florid and/or sclerosing adenosis (FA+SA), papillomatosis (PM), apocrine metaplasia (AM), typical ductal hyperplasia (TDH) and atypical ductal hyperplasia (ADH) in 57 specimens collected at 1 cm, in 50 at 2 cm and in 35 at 3 cm from the autologous BC. 23 BL displayed the same morphological features. TDH+ADH combined to FA were found in 5 PTT at 1cm, in 5 at 2 cm, and in 3 at 3 cm whereas none of the benign tumors presented these combinations of morphological changes. TDH was associated to AM, SA, PM in 10 PTT closest to the autologous BC, in 10 and in 5 PTT sampled at 2 and 3 cm respectively. Only two benign tumors displayed these histopathological aspects. In addition, 19 fibroadenomas (FBA),4 of which combined with FA, were diagnosed.

Characteristics	**Total number of patients = 72**	
	N°	**%**
Age = 51.6 (range 36-88)	-	-
Tumoral histotype		
Infiltrating Ductal Carcinoma	62	86.1
Infiltrating Lobular Carcinoma	10	13.9
Tumor size		
T1	39	54.1
T2	31	43.1
T3	2	2.8
Nodal Status		
N_0	41	56.9
N_1	31	43.1
ER (cut off > 10%)		
Positive	48	66.6
Negative	24	33.4
PgR (cut off > 10%)		
Positive	49	68.1
Negative	23	31.9
HER2°		
Positive	19	26.3
Negative	53	73.6
P53* (cut off > 10%)		
Positive	32	44.4
Negative	40	55.6
Fas		
Positive	31	43.1
Negative	41	56.9
FasL		
Positive	39	54.2
Negative	33	45.8

Table 7. Clinicopathological and biologic characteristics of 72 breast cancer patients

	1 cm PTT	2 cm PTT	3 cm PTT	Benign lesions
FC + FA and/or SA	41	35	27	18
FC + PM + SA + AM + FA	1	0	1	3
FC + TDH + AM	10	8	6	0
FC + ADH + FA + PM	3	4	1	1
FC + AM + SA	2	3	0	1
TDH + ADH +FA	5	5	3	0
TDH + AM + SA + PM	10	10	5	2
FBA	0	0	0	15
FBA + FA	0	0	0	4
Total	**72**	**65**	**43**	**44**

Table 8. Pathological features of multiple peritumoral tissues and benign lesions

3.2. HER2 and p53 expression in breast cancers, multiple PTT and benign mammary lesions

As summarized in table 9, we evaluated HER2 and p53 expression in 44 benign tumors, 72 cancers and the corresponding PTT sampled at 1 cm (72 cases), 2 cm (65 cases) and 3 cm (43 cases) respectively from the autologous BC. Of the 44 benign lesions (4.5%), 2 were HER2 positive showing a 2+ immunostaining clearly restricted to the cell membrane. HER2 overexpression was mainly found in FA and hyperplasic areas.

Nineteen of the 72 breast cancer samples (26.3%) were HER2 positive (2+/3+ score). In 10 PTT independently of the distance from the primary tumor we observed a distinct cell membrane immunostaining prevalently confined to TDH, ADH and FA. In these PTT we never found a 3+ score immunostaining whereas a weak 1+ score staining was observed in 9 PTT (12.5%) collected at 1 cm, 6 at 2 cm (9.2%) and 5 at 3 cm (11.6%).

The percentage of HER2 overexpression was significantly higher in malignant tumor than in benign lesions ($p=0.007$) and PTT sampled at 1, 2 and 3 cm ($p=0.01$, $p=0.001$ and $p= 0.03$ respectively). No difference in HER2 overexpression was observed among the three PTT analyzed by IHC ($p=0.49$, $p=0.99$ and $p=0.99$ respectively). HER2 immunostaining in tumor and PTT samples are shown in figure 22A and 22B. Smooth muscle actin staining was also performed to verify differentiation of BC and PTT (figure 23A and 23B).

	HER2			P53 Positive cases (%)	HER2 + p53 Positive cases (%)
	1+ (%)	2+ (%)	3+(%)		
Benign lesions 44 patients	5 (11.4)	2 (4.5)	0 (0.0)	3 (6.8)	1(2.2)
Breast cancers 72 patients	9 (12.5)	11 (15.3)	8 (11.1)	34 (47.2)	12 (16.6)
PTT 1 cm 72 patients	9 (12.5)	5 (6.9)	0 (0.0)	5 (6.9)	4 (5.5)
PTT 2 cm 65 patients	6 (9.2)	3 (4.6)	0 (0.0)	1 (1.5)	1 (1.5)
PTT 3 cm 43 patients	5 (11.6)	2 (4.6)	0 (0.0)	1 (2.3)	0

Table 9. Evaluation of the HER2 and p53 expression in 44 benign mammary lesions, 72 breast cancer and autologous peritumoral tissues

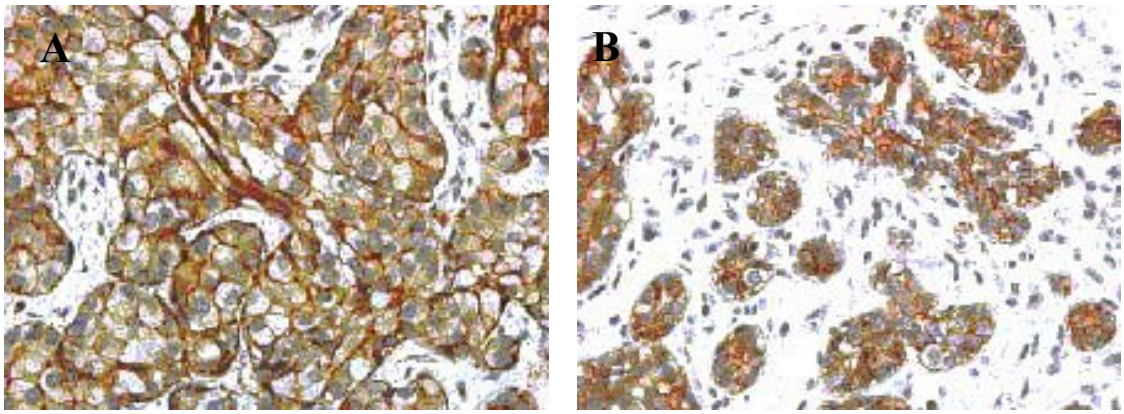

Figure 22. HER2 positivity in breast cancer (A) and PTT sample (B)

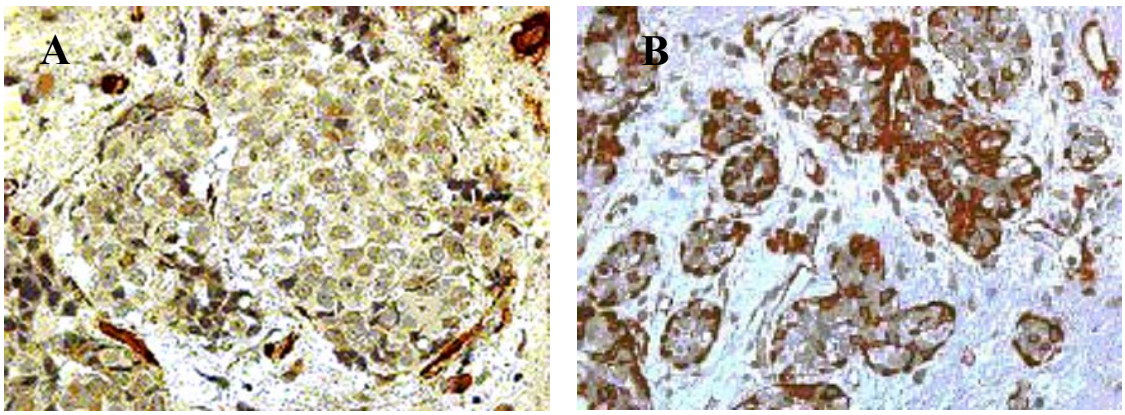

Figure 23. Loss of myoepithelial cells in the invasive carcinoma (C) and maintenance of myoepithelial cells (D) in the correspondent PTT

Thirty-four BC (47.2%) were p53 positive (figure 24A) with variable percentage of nuclear staining ranging from 10% to 90% with a median value of 40%. In contrast we observed p53 nuclear accumulation in only 1 benign lesion. Also in PTT, p53 positivity was seen in a limited number of lesions (figure 24B). 4 cases of TDH and 3 FA displayed p53 nuclear accumulation in a low percentage of epithelial cells ranging from 1% to 10%.

p53 expression was higher in malignant tumors with respect to benign lesions ($p<0.001$) and in malignant tumors versus 1, 2 and 3 cm PTT ($p<0.0001$). No significant difference in p53 positivity was observed among the three PTT ($p=0.21$, $p=0.42$ and $p=0.99$ respectively).

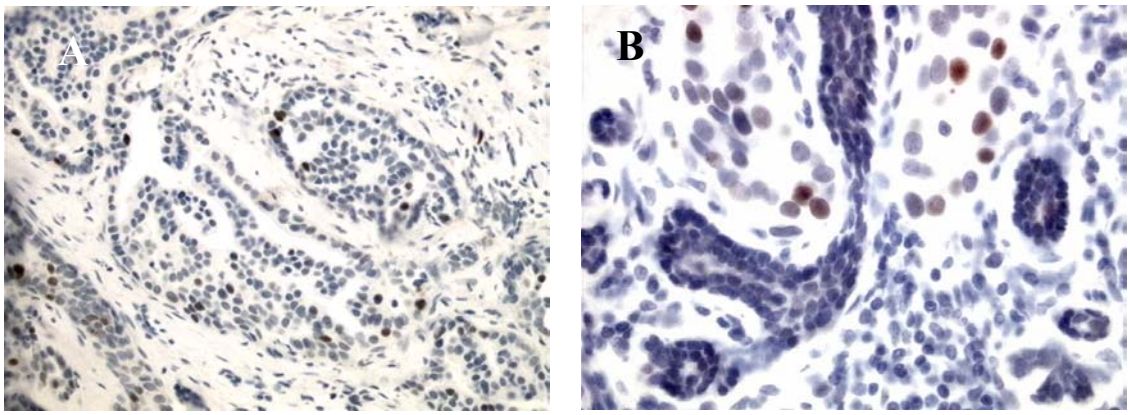

Figure 24. p53 immunostaining in breast cancer (A) and PTT sample (B)

A concomitant expression of HER2 and p53 was detected in 1 benign lesion (TDH), 12 BC and 5 PTT (3 FA, 1 TDH and 1 ADH), 4 of which were collected at 1 cm from autologous BC and 1 at 3 cm.

In order to verify whether in PTT HER2 overexpression, detected by the means of IHC methods, was correlated to gene amplification, we submitted to FISH analysis the 10 PTT immunohistochemically positive for HER2 (figure 25). Table 10 shows that 5 (2 FA, 3 TDH) out of the 10 PTT demonstrated HER2 gene amplification (ratio >2). In the autologous BC HER2 was amplified in 7 of these 10 cases.

	Histotype		**HER2 status**			
	Tumor	**PTT**	**Tumor**		**PTT**	
			FISH	**IHC**	**FISH**	**IHC**
Patient 1	IDC	FA	1.1	2+	1.3	2+
Patient 2	IDC	FA	2.3	2+	1.1	2+
Patient 3	LC	TDH	1.2	2+	1.2	2+
Patient 4	IDC	FA	4.6	3+	7.6	2+
Patient 5	IDC	FC +FA+ TDH	7.5	3+	5.1	2+
Patient 6	IDC	FC + SA	3.7	3+	1.8	2+
Patient 7	IDC	FA	5.2	3+	3.4	2+
Patient 8	IDC	TDH + AM + PM	1.5	2+	2.4	2+
Patient 9	IDC	FC + AM + TDH	2.8	2+	2.2	2+
Patient 10	IDC	FC	2.1	2+	1.4	2+

Table 10. Comparison between HER2 IHC overexpression and gene amplification in breast cancer and autologous multiple peritumoral tissues

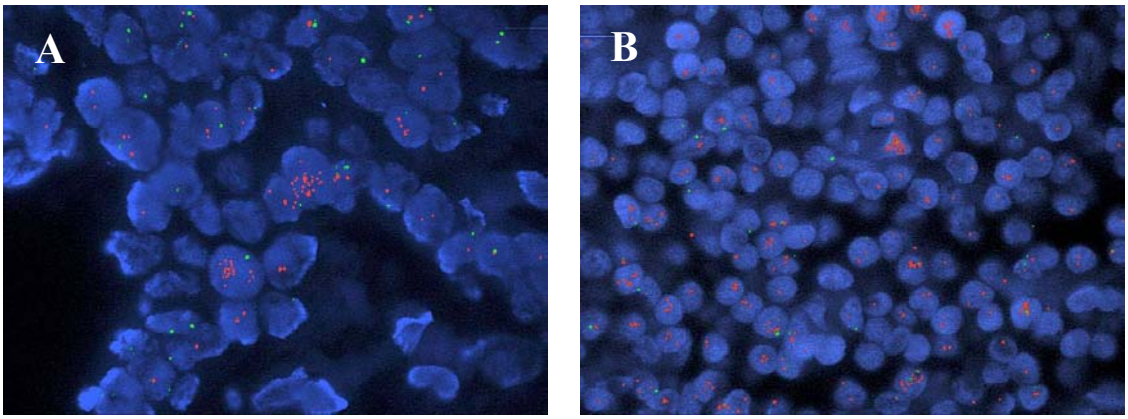

Figure 25. HER2 gene amplification in breast cancer (A) and PTT (B) sample by FISH analysis

3.3. Fas and FasL expression in benign mammary lesions, breast cancers and multiple PTT

Figure 26 shows loss of Fas reactivity in breast cancer and Fas positivity in PTT samples. Table 11 reports that 40 out of 44 benign lesions (90.9%) showed a strong and homogeneous Fas expression prevalently localized on cell membrane whereas only 22% of benign tumors were FasL positive. On the other hand, when the 72 malignant tumors were evaluated, only 41 BC (56.9%) showed Fas positivity that was often heterogeneous in intensity and cell distribution whereas FasL (figure 27) was positive in 45.8% of the cases. The rate of cases expressing Fas was significantly lower in BC than in BL ($p<0.0001$) as well as the rate of FasL positive cases was significantly higher in BC than in BL ($p<0.001$). In both cases tumor-infiltrating lymphocytes provided internal controls. In BC the expression of receptor and ligand antigens appeared to be inversely related ($p<0.0001$) with 37.5% of Fas+/Fas-L– and 26.4% Fas–/Fas-L+. Double positive (Fas+/Fas-L+) and double negative (Fas–/Fas-L–) phenotypes accounted for 19.4% and 16.7% respectively.

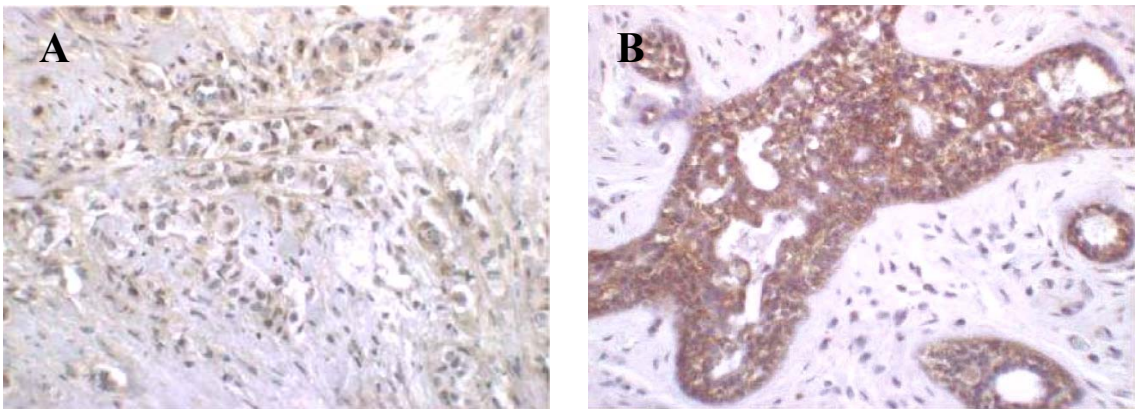

Figure 26. Loss of Fas reaction in breast cancer (A) and Fas positivity in PTT sample (B)

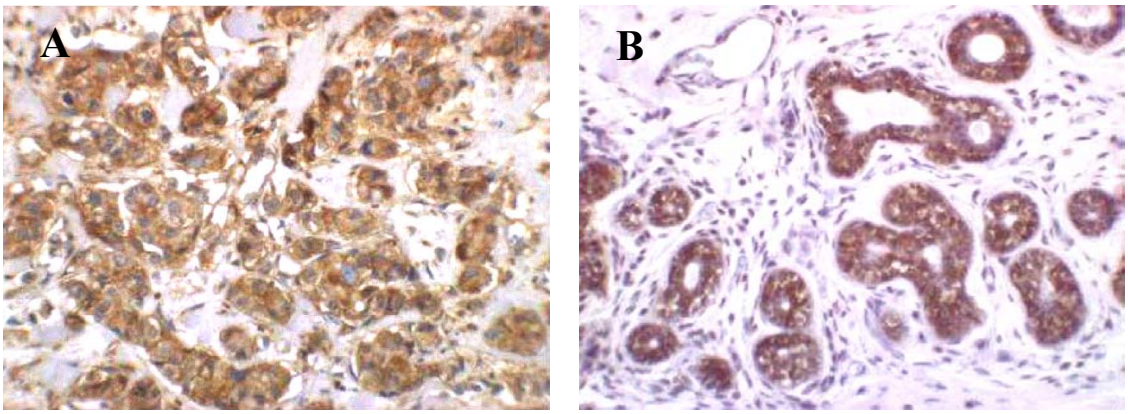

Figure 27. FasL immunostaining in breast cancer (A) and PTT sample (B)

Antigenic phenotype		Benign lesions 44 pts	%	Fas positivity (%)	FasL positivity (%)
Fas+	**FasL+**	8	18.1		
Fas-	**FasL+**	2	4.5		
Fas+	**FasL-**	32	72.9	90.9	22.7
Fas-	**FasL-**	2	4.5		
		Breast cancer 72 pts		**Fas positivity (%)**	**FasL positivity (%)**
Fas+	**FasL+**	14	19.4		
Fas-	**FasL+**	19	26.4		
Fas+	**FasL-**	27	37.5	56.9	45.8
Fas-	**FasL-**	12	16.7		
		PTT 1 cm 72 pts		**Fas positivity (%)**	**FasL positivity (%)**
Fas+	**FasL+**	23	31.9		
Fas-	**FasL+**	7	9.7		
Fas+	**FasL-**	40	55.5	87.5.3	41.6
Fas-	**FasL-**	2	2.8		
		PTT 2 cm 65 pts		**Fas positivity (%)**	**FasL positivity (%)**
Fas+	**FasL+**	15	23.1		
Fas-	**FasL+**	3	4.6		
Fas+	**FasL-**	44	67.7	90.8	27.7
Fas-	**FasL-**	3	4.6		
		PTT 3 cm 43 pts		**Fas positivity (%)**	**FasL positivity (%)**
Fas+	**FasL+**	8	18.7		
Fas-	**FasL+**	2	4.6		
Fas+	**FasL-**	31	72.1	90.7	23.3
Fas-	**FasL-**	2	4.6		

Table 11. Evaluation of the contemporary expression of Fas and FasL in multiple tissue samples from 44 patients with benign mammary lesions and 72 patients with breast cancer

The percentage of Fas expression in normal appearing breast epithelium in the three different samples adjacent to invasive cancer was similar to that observed in BL independently of the distance from the autologous BC (90.9% vs 87.5% at 1 cm, 90.8% at 2 cm, 90.7% at 3 cm). In contrast, FasL was significantly upregulated in PTT sampled at 1 cm with respect to BL (22.7% p=0.05). Therefore the percentage of FasL positive cases in 1cm PTT (41.6%) was similar to that found in BC (45,8%) and no statistically significant difference was evidenced between invasive cancer and the closest PTT (p=0.73). FasL expression in breast specimens collected farther from the autologous BC was similar to that observed in BL (22.7% vs. 27.7% at 2 cm, 23.3% at 3 cm) and significantly different from BC (p= 0.04 at 2 cm and p=0.02 at 3 cm).

3.4. COX2 expression in breast cancer and metastatic lymph node specimens

Clinicopathological characteristics of patients of the COX2, hormone receptors, HER2, p53, Ki67, Fas, FasL, PGE_2S and EGFR investigations are shown in table 12.

Characteristics	Total number of patients = 186	
	N°	%
Age = 48.12 (range 25-66)	-	-
Tumor size		
<2 cm	183	98.4
>2 cm	3	1.6
Histotype		
Ductal carcinoma	154	82.8
Lobular carcinoma	32	17.2
Nodal Status		
N_o	91	48.9
N_1	95	51.1
ER (cut off > 10%)		
Positive	94	50.5
Negative	92	49,5
PgR (cut off > 10%)		
Positive	103	55.4
Negative	83	44.6
HER2°		
Positive	74	39.8
Negative	112	60.2
P53		
Positive	76	40.9
Negative	110	59.1
COX2		
Positive	147	79.0
Negative	39	21.
PGE_2S		
Positive	101	83.5
Negative	20	16.5
EGFR		
Positive	82	49.1
Negative	85	50.9
Fas		
Positive	107	57.5
Negative	79	42.5
FasL		
Positive	91	48.9
Negative	95	51.1
Relapse		
Positive	60	32.2
Negative	126	67.8
Cancer-related death		
Alive	148	81.7
Dead	33	18.3

Table 12. Clinicopathologic and biologic characteristics of 186 breast cancer patients

Patients had a mean age of 48.12 years (range 25-66), including pre- and postmenopausal women. Of the 186 breast carcinomas 154 were infiltrating ductal carcinoma

and 32 infiltrating lobular carcinoma. Tumor sizes varied from T_1 to T_4 (T_1 in 98 cases, T_2 in 85 cases, T_3 in 2 case and T_4 in 1 case). Ninety-one patients were node negative while 95 tumors had already nodal metastases. Estrogen receptor positivity was found in 94 cases and 103 tumors were progesterone receptor positive. Seventy-four malignant lesions overexpressed HER2 and 76 proved to be p53 positive. COX2 was expressed in 147 breast cancer specimens while 39 cases were COX2 negative. PGE_2S positivity occurred in 101 cases out of the 121, while 82 of the 167 samples investigated expressed EGFR. Fas positivity occurred in 107 cases and 91 lesions showed FasL reactivity. Sixty patients had relapse and 33 patients died of the disease.

COX2 immunoreactivity in primary tumors (figure 28A) and in metastatic lymph node samples (figure 28B) is described in table 13. Of the 82 COX2 positive primary tumor, 81 (98.8%) had COX2 positive metastatic nodes while in 1 case (1.2%) nodal metastasis was COX2 negative. Among the 13 COX2 negative metastatic primary tumor, 9 (69.2%) had COX2 positive metastatic nodes, while in 4 cases (30.8%) we found no COX2 expression in the metastatic lymph nodes. Altogether, increase in expressing COX2 was found in metastatic lymph nodes regarding primary tumors (93.7% *vs* 86.5%).

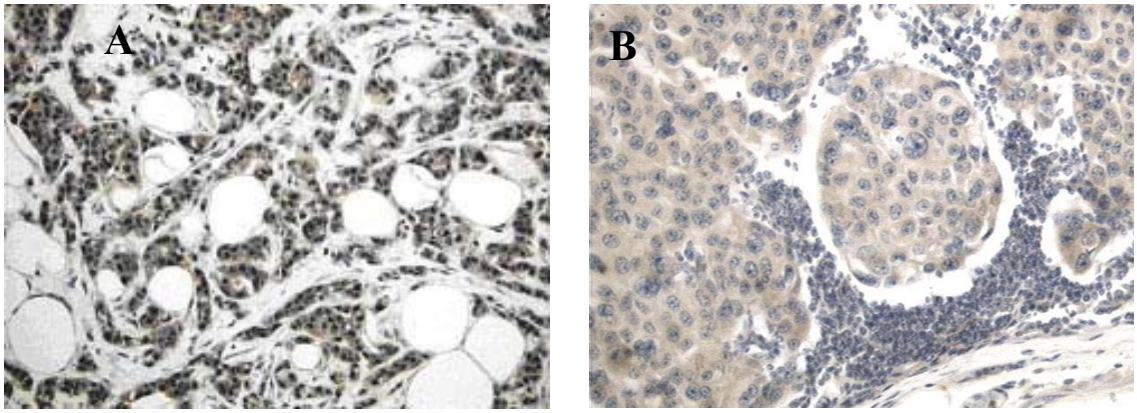

Figure 28. COX2 positivity in breast cancer (A) and in metastatic lymph node (B)

N° of Patients	Primary Tumor	Lymph nodes
81	+	+
1	+	-
9	-	+
4	-	-
Total (95)	**82 (84.3%)**	**91 (95.7%)**

Table 13. COX2 reactivity in breast cancer samples and their metastatic lymph nodes

Table 14 shows the increased COX2 expression among metastatic lymph nodes versus primary tumors too. Of the 10 patients with 1 metastatic node each, 5 had COX2 positive primary tumor and 7 had COX2 positive metastases. Among the 15 patients, each with 2 metastatic nodes, all primary tumors expressed COX2 and only 1 metastatic node was found to be COX2 negative. Three metastatic nodes per breast cancer were found in 21 patients, of which 19 primary tumors expressed COX 2 and 20 cases exhibited COX2 positive nodes. Ten patients had 4 metastatic nodes each, 9 of them being COX2 positive in primary tumor and 10 cases being COX2 positive regarding the metastatic nodes. Seven of the 8 cases with 5 metastatic lymph nodes each were found to express COX2 in the primary tumor and all the cases exhibited COX2 positive metastases. Among the 11 patients, each having 6 metastatic nodes, 10 were found to have COX2 primary tumors and all the cases had COX2 positive metastases. Of the 5 cases with 7 metastatic nodes each, 3 primary tumors were COX2 positive while all cases were COX2 positive regarding the metastatic lymph nodes. Of the 6 cases, each with 8 metastatic nodes, all cases were found to express COX2 both in primary tumors and metastatic lymph nodes. Five patients had breast cancer with 9 metastatic nodes each, 4 of them having COX2 positive primary tumor and 4 of them having COX2 positive metastases. Among the 4 cases with 10 metastatic nodes each, all primary tumors and metastases expressed COX2.

	N° of Patients	**Primary Tumor COX2+**	**N° of metastatic lymph nodes**	**N° of cases with COX2 + metastatic lymph nodes**
	10	5	1	7
	15	15	2	14
	21	19	3	20
	10	9	4	10
	8	7	5	8
	11	10	6	11
	5	3	7	5
	6	6	8	6
	5	4	9	4
	4	4	10	4
Total	**95**	**82 (84.8%)**		**89 (93.7%)**

Table 14. COX2 immunoreactivity in primary tumors and their metastatic lymph node samples

3.5. PGE_2S expression in breast cancer cases

As described in table 15, only 121 primary breast cancer cases had tissue samples available for PGE_2S analysis. Of the 102 COX2 positive samples 94 (92.2%) showed PGE_2S immunoreactivity, 8 cases (7.8%) were PGE_2S negative. Among the 19 cases negative for COX2 expression, 7 cases (36.8%) showed PGE_2S positivity and 12 cases (63.2%) did not express PGE_2S. No metastatic lymph node tissue samples were available for PGE_2S immunohistochemical reaction. PGE_2S immunostaining is shown in figure 29A.

COX2 in primary tumor	PGE_2S in primary tumor +	PGE_2S in primary tumor -	Total
+	94 (92.2%)	8 (7.8%)	102 (100.0%)
-	7 (36.8%)	12 (63.2%)	19 (100.0%)

Table 15. COX2 and PGE_2S reactivity in 121 breast cancer patients

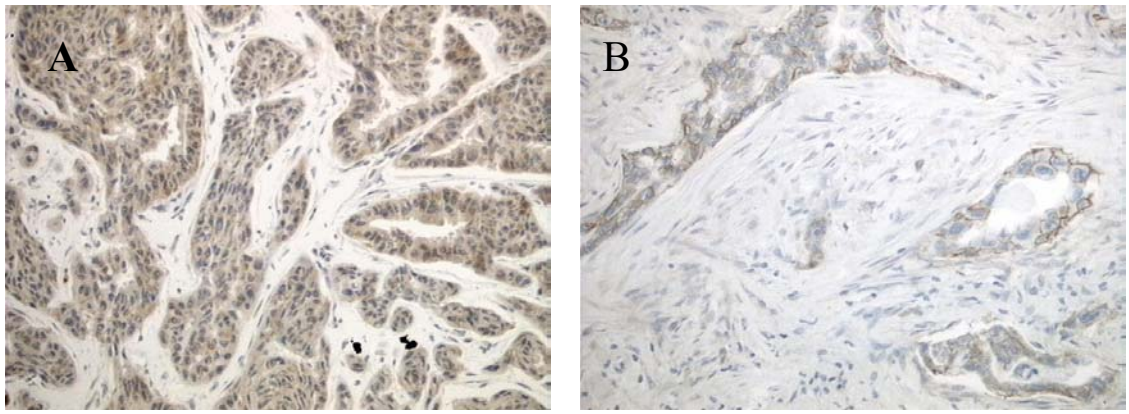

Figure 29. PGE_2S positivity (A) and EGFR immunostaining in breast cancer (B)

3.6. EGFR expression in breast cancer cases

Table 16 shows the results of EGFR immunostaining (figure 29B). Among the 186 primary breast cancer cases 167 cases had tissue samples available for EGFR expression analysis. During the immunohistochemical analyses, 66 (46.5%) of the 142 COX2 positive samples expressed EGFR, while 76 samples (53.5%) were EGFR negative. Of the 25 samples showing no COX2 reactivity, 16 (64.0%) were EGFR positive and 9 samples (36.0%) were EGFR negative. No metastatic lymph node samples were available for further analysis.

COX2 expression	EGFR expression +	-	Total
+	66 (46.5%)	76 (53.5%)	142 (100.0%)
-	16 (64.0%)	9 (36.0%)	25 (100.0%)

Table 16. COX2 and EGFR reactivity in 167 breast cancer patients

3.7. Statistical analysis

As shown on figure 30A and 30B, distinguishing COX2 positivity in primary tumors as score 1,2 and 3 caused no significant difference in impact on DFS when compared to scoring COX2 staining only positive and negative (p=0.017 vs. p=0.002). Thus, we considered COX2 expression only positive or negative in the followings.

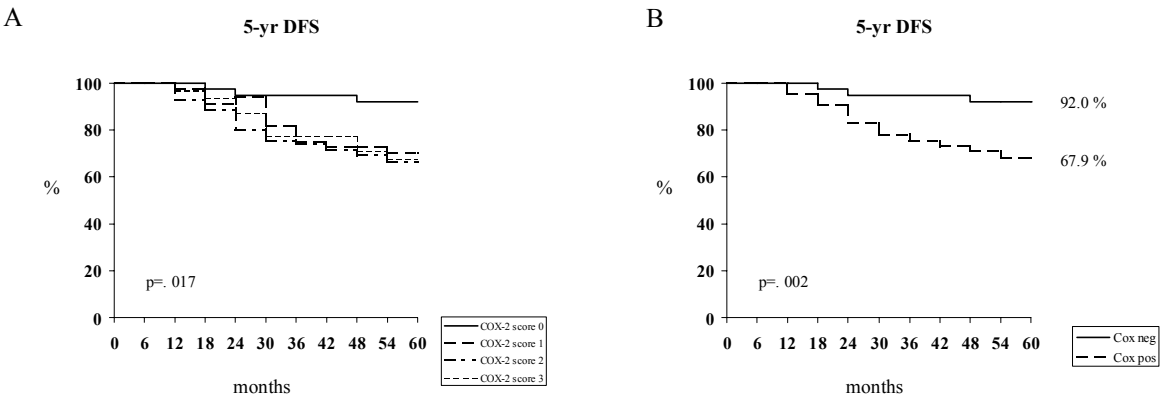

Figure 30. Impact of COX2 expression on DFS (scored 1/2/3 *vs* scored +/-)

Relationships between COX2, PGE_2S expression and biopathological variables are shown in table 17. COX2 expression was significantly related to nodal status (p=0.01), Ki67 expression (p=0.009) and relapse (p=0.03) while no significant relationship was found between COX2 expression and tumor size (p=0.93), tumor histotype (p=0.73), ER status (p=0.64), PgR status (p=0.35), HER2 expression (p=0.63), p53 status (p=0.15) and cancer-related death (p=0.25). PGE_2S activity was significantly related to COX2 expression (p<0.0001) and relapse (p=0.03), borderline significance was found between PGE_2S positivity and nodal status (p=0.08), ER status (p=0.07) and Ki67 expression (p=0.089), while no relationship was found

between PGE_2S expression and tumor size (p=0.30), tumor histotype (p=0.65), PgR status (p=0.60), HER2 expression (0.12), p53 status (p=0.37) and cancer-related death (p=0.18).

		Frequency of immunoreactive cases				
Variable	**No. of cases (186)**	**COX2 (%)**	**p value**	**No. of cases (121)**	**PGE_2S (%)**	**p value**
Tumor size						
< 2 cm	99	78.8	0.93	66	80.3	0.30
> 2 cm	87	79.3		55	87.3	
Histotype						
Ductal	154	78.6	0.73	101	84.2	0.65
Lobular	32	81.3		20	80.0	
Nodal status						
N_0	90	71.1	0.01	57	77.2	0.08
N_+	96	86.5		64	89.0	
ER status						
Negative	92	80.4	0.64	65	89.2	0.07
Positive	94	77.7		56	76.8	
PgR status						
Negative	83	75.9	0.35	61	85.2	0.60
Positive	103	81.6		60	81.7	
HER2/*neu*						
Negative	112	83.9	0.63	72	79.2	0.12
Positive	74	86.5		49	89.8	
p53 status						
Negative	110	75.5	0.15	74	81.1	0.37
Positive	76	84.2		47	87.2	
COX2						
Negative	–	–	–	27	48.1	<0.0001
Positive				94	83.6	
Ki67						
<30	109	72.5	0.009	70	78.6	0.089
>30	77	88.3		51	90.2	
Relapse						
Negative	126	81.0	0.03	77	77.9	0.03
Positive	60	93.3		44	93.2	
Cancer-related death						
Alive	145	84.1	0.25	93	80.6	0.18
Dead	36	91.7		25	92.0	

Table 17. Relationship between COX-2, PGE_2S expression and biopathological variables

Tables 18 and 19 summarize the results of univariate and multivariate analyses of prognostic factors for DFS and OS respectively.

Tumor size (OR=2.10, CI=1.25-3.51, p=0.05), nodal status (OR=3.76, CI=2.11-6.69, p<0.001), COX2 expression (OR=4.39, CI=1.59-12.11, p=0.004), PGE_2S positivity (OR=3.36 (CI=1.04-10.85, p=0.004), Ki67 expression (OR=1.95, CI=1.18-3.24, p=0.009), Fas positivity (OR=0.18, CI=0.10-0.31) and FasL expression (OR=3.28, CI=1.90-5.66) were found to be significant predictors of DFS in univariate analysis, while a borderline significance was found between DFS and p53 positivity (OR=1.60, CI=0.96-2.65, p=0.069). ER status (HR=1.02, CI=0.62-1.69, p=0.93), PgR expression (HR=1.06, CI=0.64-1.76, p=0.83) and HER2 positivity (OR=1.01, CI=0.59-1.71) had no significant impact on DFS. When considering concomitant expression of different variables, patients with tumors expressing both COX2 and p53 (OR=5.20, CI=1.58-17.13, p=0.007) or COX2 and PGE_2S (OR=7.96, CI=1.09-57.96, p=0.04) were found to have significantly reduced DFS (data not shown).
Tumor size (OR=2.17, CI=1.18-3.98, p=0.013), nodal status (OR=4.61, CI=2.16-3.83, p<0.001), COX2 expression (OR=3.50, CI=1.07-11.46, p=0.039), Ki67 positivity (OR=5.56, CI=1.71-18.09), Fas expression (OR=0.09, CI=0.03-0.29) and FasL expression (OR=1.96, CI=1.19-3.32) were significant predictors of DFS even in multivariate analyses.

Predictors of OS are shown in table 19. In univariate analysis, tumor size (OR=2.38, CI=1.20-4.70, p=0.013), nodal status (OR=3.89. CI=1.77-8.55, p=0.001), p53 positivity (OR=2.17, CI=1.12-4.21, p=0.02), Ki67 positivity (OR=2.43, CI=1.25-4.75, p=0.009), HER2 expression (2.68, CI=1.48-4.86), Fas positivity (OR=0.07, 0.03-0.16) and FasL expression (OR=6.19, CI=3.05-12.53) were significant predictors of OS, while a borderline significance was found between COX2 expression and OS (HR=3.15, CI=0.87-10.27, p=0.05). Histotype (OR=1.65, CI=0.70-3.89), PGE_2S positivity (OR=2.66, CI=0.63-11.29, p=0.19), EGFR expression (OR=1.49, 0.79-2.82), ER status (OR=1.39, CI=0.72-2.69, p=0.33) and PgR status (OR=1.51, CI=0.79-2.91, p=0.22) had no significant impact on OS.
Similarly to DFS, concomitant expression of COX2 and p53 in the primary tumors significantly influenced OS (OR=4.71, CI1.10-20.16, p=0.037), while in contrary to DFS, patients with tumors expressing both COX2 and PGE_2S had not significantly reduced OS (OR=3.80, CI=0.51-28.25, p=0.19) (data not shown).
In multivariate analysis, nodal status (OR=3.66, CI=1.66-8.07, p=0.001), HER2 positivity (OR=4.86, CI=1.12-20.99) and Fas expression (OR=0.03, CI=0.005-0.17) were significant predictors of OS, while a borderline significance was found between OS and tumor size (OR=1.87, CI= 0.93-3.79, p=0.081), p53 positivity (OR=1.89, CI=0.96-3.74, p=0.068), Ki67 positivity (OR=1.83, CI=0.91-3.68, p=0.089) and FasL expression (OR=4.29, CI=0.97-

18.88). Histotype (OR=2.37, CI=0.34-16.96), COX2 expression (OR=0.14, CI=0.02-1.26), PGE_2S positivity (OR=1.73, CI=0.18-16.7), EGFR expression (OR=0.88, CI=0.20-3.83), ER status (OR=0.29, CI=0.05-1.70) and PgR status (OR=2.7, CI=0.48-15.4) had no influence on OS.

	Univariate analysis		Multivariate analysis	
Variable	**OR (95% CI)**	**p value**	**OR (95% CI)**	**p value**
Tumor size (< 2 cm vs. > 2 cm)	2.10 (1.25-3.51)	0.005	2.17 (1.18-3.98)	0.013
Nodal status (N_0 *vs.* N_+)	3.76 (2.11-6.69)	<0.001	4.61 (2.16-3.83)	<0.001
Histotype (ductal vs. lobular)	0.89 (0.45-1.75)	0.78	0.89 (0.25-3.19)	0.88
COX2 (positive vs. negative)	4.39 (1.59-12.11)	0.004	3.50 (1.07-11.46)	0.039
PGE_2S expression (positive vs. negative)	3.36 (1.04-10.85)	0.004	2.96 (0.69-12.7)	0.22
EGFR expression (positive vs. negative)	1.44 (0.80-2.59)	0.37	0.84 (0.31-2.32)	0.78
p53 status (positive vs. negative)	1.60 (0.96-2.65)	0.069	2.63 (0.90-7.71)	0.14
Ki67 status (positive vs. negative)	1.95 (1.18-3.24)	0.009	5.56 (1.71-18.09)	0.019
ER status (positive vs. negative)	1.02 (0.62-1.69)	0.93	0.42 (0.09-1.87)	0.34
PgR status (positive vs. negative)	1.06 (0.64-1.76)	0.83	3.35 (0.75-15.08)	0.19
HER2/*neu* (positive vs. negative)	1.01 (0.59-1.71)	0.96	0.61 (0.20-1.93)	0.48
Fas (positive vs. negative	0.18 (0.10-0.31)	<0.001	0.09 (0.03-0.29)	0.001
FasL (positive vs. negative)	3.28 (1.90-5.66)	<0.001	1.96 (1.19-3.32)	0.01

Table 18. Univariate and multivariate analyses of prognostic factors for DFS in 186 breast cancer patients

Variable	Univariate analysis		Multivariate analysis	
	OR (95% CI)	p value	OR (95% CI)	p value
Tumor size (<2 cm vs. >2 cm)	2.38 (1.20-4.70)	0.013	1.87 (0.93-3.79)	0.081
Nodal status (N_0 vs. N_1)	3.89 (1.77-8.55)	0.001	3.66 (1.66-8.07)	0.001
Histotype (ductal vs. lobular carcinoma)	1.65 (0.70-3.89)	0.34	2.37 (0.34-16.96)	0.47
COX2 expression (positive vs. negative)	3.15 (0.87-10.27)	0.050	0.14 (0.02-1.26)	0.15
PGE_2S expression (positive vs. negative)	2.66 (0.63-11.29)	0.19	1.73 (0.18-16.7)	0.69
EGFR expression (positive vs. negative)	1.49 (0.79-2.82)	0.30	0.88 (0.20-3.83)	0.88
p53 status (positive vs. negative)	2.17 (1.12-4.21)	0.02	1.89 (0.96-3.74)	0.068
Ki67 status (positive vs. negative)	2.43 (1.25-4.75)	0.009	1.83 (0.91-3.68)	0.089
ER status (positive vs. negative)	1.39 (0.72-2.69)	0.33	0.29 (0.05-1.70)	0.25
PgR status (positive vs. negative)	1.51 (0.79-2.91)	0.22	2.7 (0.48-15.4)	0.35
HER2/*neu* (positive vs. negative)	2.68 (1.48-4.86)	0.007	4.86 (1.12-20.99)	0.08
Fas (positive vs. negative	0.07 (0.03-0.16)	<0.001	0.03 (0.005-0.17)	0.002
FasL (positive vs. negative)	6.19 (3.05-12.53)	<0.001	4.29 (0.97-18.88)	0.109

Table 19. Univariate and multivariate analyses of prognostic factors for OS in 186 breast cancer patients

Impact of COX2 expression on DFS (p=0.002) can also be seen on figure 30B showing the Kaplan-Meier curves for 186 breast cancer patients. Figure 31A and 31B demonstrate the impact of p53 positivity on DFS (p=0.067) and OS (p=0.02) according to the Kaplan-Meier curves, while impact of concomitant expression of COX2 and p53 expression on DFS (p=0.003) and on OS (p=0.02) can be seen on figure 31C and 31D demonstrating the respective Kaplan-Meier curves.

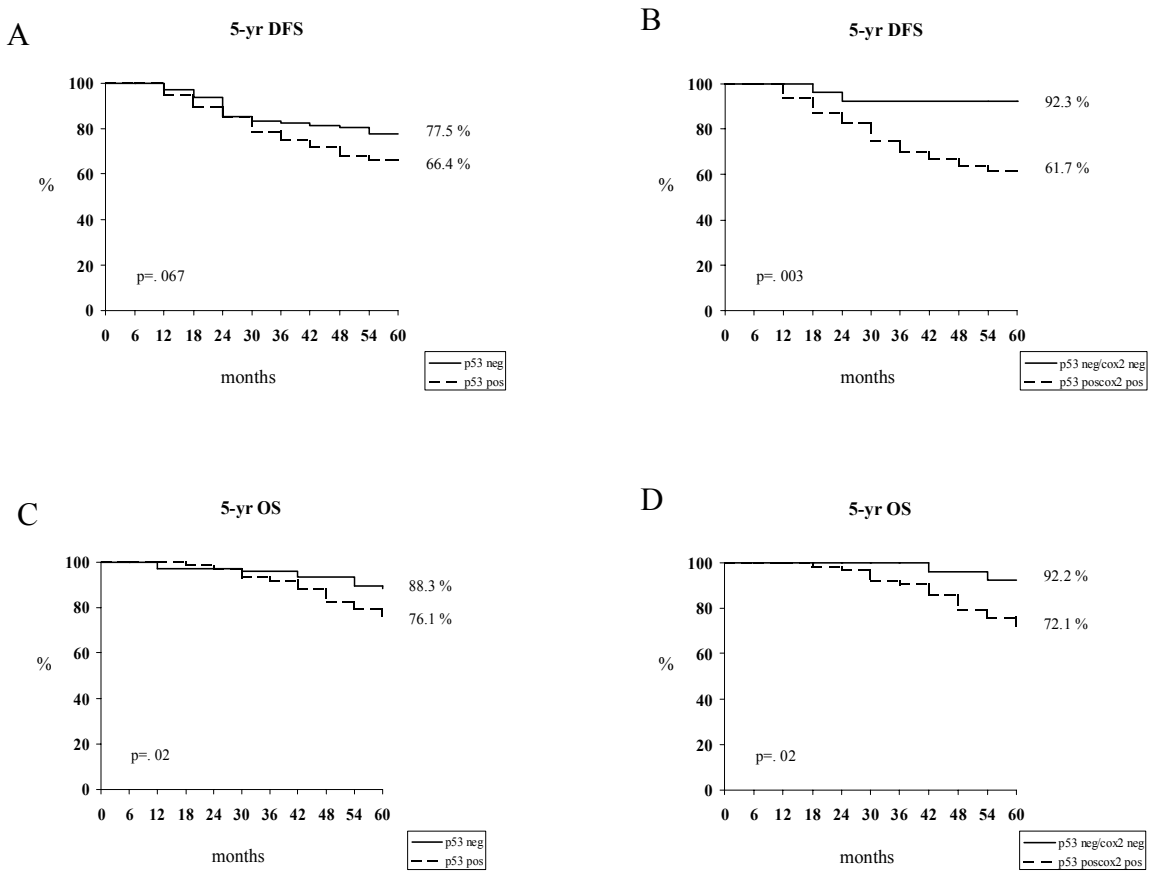

Figure 31. Impact of p53 expression on DFS (A) and OS (C), and concomitant COX2/ p53 expression on DFS (B) and OS (D)

Results of determining relationships between prognostic factors are shown in table 20. COX2 expression in primary breast tumors was significantly related to p53 expression (OR=2.917, CI=1.308-6.505) and Ki67 expression (OR=1.993, CI=1.001-3.970). There was a significant relationship between HER2 and FasL (OR=1.687, CI=1.026-2.774), Ki67 (OR=1.765, CI=1.068-2.916) and EGFR (OR=2.314, CI=1.320-4.056) positivity. ER expression was significantly related to Ki67 (OR=0.433, CI=0.264-0.710), p53 (OR=0.427, CI=0.258-0.706), PgR (OR=99.171, CI=40.558-242.489) and EGFR (OR=0.448, CI=0.255-0.786) expression, while PgR expression showed significant relationship to Ki67 (OR=0.360, CI=0.217-0.596), EGFR (OR=0.377, CI=0.214-0.664) and p53 (OR=0.481, CI=0.292-0.792) expression. p53 expression was significantly related to FasL (OR=1.843, CI=1.122-3.028) and to Ki67 (OR=2.540, CI=1.524-4.232) expression, while there was a significant relationship also between Fas and FasL expression (OR=0.245, CI=0.146-0.411).

Variable I	Variable II	OR	5% CI	95% CI
FAS	FASL	0.245	0.146	0.411
Ki67	FASL	0.923	0.569	1.498
EGFR	FASL	0.935	0.575	1.730
PGE2S	FASL	1.306	0.595	2.866
COX2	FASL	1.333	0.675	2.633
p53	FASL	1.843	1.122	3.028
HER2	FASL	1.687	1.026	2.774
PgR	FASL	0.966	0.595	1.570
ER	FASL	1.001	0.618	1.622
Ki67	FAS	1.245	0.763	2.031
EGFR	FAS	1.094	0.628	1.904
PGE2S	FAS	0.831	0.378	1.825
COX2	FAS	1.019	0.515	2.014
p53	FAS	0.781	0.476	1.282
HER2	FAS	1.040	0.632	1.713
PgR	FAS	0.818	0.500	1.338
ER	FAS	0.833	0.511	1.358
EGFR	Ki67	1.226	0.703	2.136
PGE2S	Ki67	0.885	0.407	1.928
COX2	Ki67	1.993	1.001	3.970
p53	Ki67	2.540	1.524	4.232
HER2	Ki67	1.765	1.068	2.916
PgR	Ki67	0.360	0.217	0.596
ER	Ki67	0.433	0.264	0.710
PGE2S	EGFR	0.770	0.304	1.948
COX2	EGFR	1.873	0.845	4.155
p53	EGFR	1.435	0.818	2.515
HER2	EGFR	2.314	1.320	4.056
PgR	EGFR	0.377	0.214	0.664
ER	EGFR	0.448	0.255	0.786
COX2	PGE2S	$>1.7 \times 10^7$	$>1.7 \times 10^7$	$>1.7 \times 10^7$
p53	PGE2S	1.714	0.753	3.905
HER2	PGE2S	0.000	0.000	0.000
PgR	PGE2S	1.275	0.586	2.776
ER	PGE2S	0.000	0.000	0.000
HER2	COX2	1.226	0.608	2.471
PgR	COX2	0.919	0.465	1.816
ER	COX2	0.515	0.256	1.036
p53	COX2	2.917	1.308	6.505
HER2	p53	1.417	0.859	2.336
PgR	p53	0.481	0.292	0.792
ER	p53	0.427	0.258	0.706
PgR	HER2	0.697	0.424	1.145
ER	HER2	0.882	0.539	1.444
ER	PgR	99.171	40.558	242.489

Table 20. Relationship of the investigated biopathological variables detemined by OR

4. DISCUSSION

The observation that small invasive BC, in some cases, are not preceded by atypical hyperplasia or in situ carcinoma, usually present in the earliest stages of breast carcinogenesis, suggests that many molecular alterations in tumor development may be morphologically silent (6, 113, 114). Therefore, the identification of biological changes occurring in normal appearing tissue surrounding invasive cancer are pivotal for understanding BC pathogenesis and for risk assessment of pre-invasive lesions. In view of observation that high levels of carcinogen DNA adducts (115), loss of retinoic acid receptor β (116) and changes in chromosome copy number (2) may occur in normal PTT, we performed the present study aimed at assessing whether breast epithelium adjacent to invasive cancer may express markers of transformation. For an easy clinical application, we chose IHC as method of analysis. Several studies have identified a number of molecular changes involving oncogenes (9) and tumor suppressor genes (117), which can increase the risk of developing invasive carcinoma in some types of pre-invasive breast lesions. In contrast, limited studies compared molecular alterations between cancer and the autologous adjacent breast tissue (10, 117, 118) and, to the best of our knowledge, no studies evaluated contemporary multiple non-involved tissues sampled from the same patient. In fact, it could be hypothesized that the closer a lesion is to invasive end-stage, the more it will resemble to the invasive cancer from a molecular point of view. Starting from this consideration, in this study we analyzed PTT sampled at 1, 2, 3 cm from the autologous tumor to ascertain whether a gradient of detectable molecular changes may exist. It is largely reported that besides proliferation, reduced apoptosis can lead to preservation of genetically aberrant cells, thus favoring initiation of breast oncogenesis (119). To this end we investigated the expression of markers relevant in both the two processes, namely HER2, a member of the EGFR family known to be overexpressed in early stages of BC (120, 121), p53, frequently mutated in invasive mammary carcinoma (117) and the interacting extracellular pro-apoptotic receptor/ligand pair, Fas/Fas-L, inversely expressed in malignant breast tumors with respect to the benign lesions (122-124). In our series of 72 BC patients HER2 was overexpressed (score 2+/3+) in 26.3% of cases. In addition, as described by other authors (6, 9, 117, 118), we found variable levels of HER2 positivity (score 1+/2+) in a low percentage of benign lesions and in some normal appearing PTT independently of the distance from the autologous BC. This immunoreactivity was prevalently detected in FA, TDH and ADH. Although alteration of HER2 status was described in a large spectrum of benign lesions (6, 9) and in non-neoplastic epithelium present on the same tissue sections as cancer (10, 117, 118), the biological significance of this HER2

immunoreactivity in non-transformed breast tissue is still a matter of debate, being mostly considered as false positive staining (125). This is in contrast with the finding that HER2 amplification can emerge *de novo* in any stage of breast carcinogenesis, may be detectable in typical as well as atypical ductal hyperplasia (10, 118, 120) and, noteworthy, women carrying benign proliferative lesions harboring amplified HER2 are at higher risk of developing cancer (9, 10). In order to better understand the biological basis of HER2 immunoreactivity in morphologically uninvolved PTT, we evaluated, by FISH analysis, gene amplification in the 10 PTT sampled at 1, 2 or 3 cm with HER2 immunostaining. Of interest, gene amplification was detected in 5 of these cases, independently from the distance from the autologous BC and in 8 out of 10 corresponding invasive cancer, demonstrating that the IHC HER2 positivity in normal-appearing breast epithelium has often underlying HER2 gene amplification.

We detected p53 nuclear staining in a limited number of benign lesions as well as in multiple PTT. The positivity, as already observed for HER2, was not related to the distance from the primary tumor and no statistical significance among specimens collected at 1, 2 and 3 cm ($p=0.21$, $p=0.42$ and $p=0.99$ respectively) was found. These results are of clinical interest since it has been recently reported that p53 immunostaining in benign epithelial cells, even if weak and focal, appears to be associated with a statistically significant increased risk of BC development (6, 126). That even a weak immunostaining is predictive of transformation, may be questionable, because p53 inactivated by mutation is usually associated to strong and diffuse nuclear accumulation (127). However, low levels of p53 protein could be a marker of cells more easily exposed to a carcinogenic microenvironment, leading to increased genetic instability and consequent tumor initiation.

Fas and FasL interactions, which play an important role in different immune functions (122-124), are crucial in the involution of the mammary epithelium preventing cellular accumulation of mutations and neoplastic transformation (128). It is largely reported (11, 13, 119, 129) that benign and malignant breast lesions are characterized by different expression of Fas and FasL molecules. In agreement with other authors (13, 129), we demonstrated that altered FasL:Fas ratio in breast carcinomas is related to adverse clinical outcome. Moreover, this is the first report in which Fas and FasL expression was accurately analyzed in malignant breast tumors and autologous normal appearing breast epithelium collected at different distance from invasive cancer. Fas expression was significantly downregulated in our series of primary tumors (130-132), but it was homogeneously expressed in PTT independently of the distance from the autologous BC (87.5%, 90.8% and 90.7% respectively). Therefore changes in Fas expression, which in the tumor may be inactivated by different molecular

mechanisms such as promoter methylation, transcriptional repression and histone acetylation (133), do not seem to be an early event in breast carcinogenesis. Although we do not know the mechanism/s underlying Fas downregulation in BC, this change may result in resistance to apoptosis i.e. accumulation of genetic changes and decrease of NK cell mediated immunosurveillance (131). Differently from Fas, FasL was significantly upregulated in malignant tumor samples (45.5%) and PTT closer (1 cm 41.7%) to the tumor with respect to BL (22.7%) and to PTT sampled at 2 cm (27.7%) and at 3 cm (23.3%) from the autologous BC. FasL in non-lymphoid tissues is known to be induced by a number of factors among which the response to activated lymphocytes has been extensively documented (134, 135). Whether this latter mechanism is responsible for FasL upregulation in about 50% of breast cancer, in 20% of benign lesions and interestingly in 41.7% of the PTT nearest to the tumor is unclear at present. One may hypothesize that in some patients FasL expression may be induced by circulating T cells recognizing MHC-peptide complexes on tumor cells. Although we cannot exclude that FasL upregulation in surrounding PTT may be induced by a paracrine mechanism (2, 136), the same immune-mediated upregulation may also occur in apparently normal breast tissues. Whatever the molecular pathways responsible for FasL expression are, this new phenotype is likely to result in being protected against T cell mediated killing, thus facilitating the accumulation of cell damages in benign lesions leading to malignant transformation.

In conclusion, our results indicate that HER2 positive breast cancers may have underlying gene amplification not only in the BC itself but also in the morphologically normal-appearing adjacent parenchyma. We have also shown that p53 nuclear accumulation can be observed in a small percentage of PTT. Neither HER2 nor p53 showed a gradient of alterations starting from the closest to the farther PTT, suggesting that these two molecules in benign tissue of cancer-containing breast could reflect a genomic damage due to long-term carcinogenic exposure. In contrast, a gradient of expression was evident for FasL since the PTT closest to invasive cancer showed an upregulated FasL and this upregulation was lost in PTT farther from the invasive carcinoma. These data support the hypothesis that FasL, in combination with other biological parameters, may be a novel biomarker useful to identify patients at higher risk of developing BC.

In women, bearing genetic or environmental risk factors, random periareolar fine-needle aspirates (FNA) may be a simple available tool to identify individuals at very high short-term risk for BC (126). In this context the completion of conventional morphological characterization with a molecular assessment using multiple biomarkers as surrogate end

points, makes cytological diagnosis less susceptible to interpretative variance. Therefore the availability of novel molecules, i.e. Fas-FasL, easy to detect by IHC in FNA, used in combination with more established parameters such as HER2 and p53, may be of particular diagnostic interest being predictive of later cancer development. To this end, larger prospective studies, aimed at establishing the clinical relevance of the present findings, are warranted.

Our results obtained by analyzing immunohistochemically 186 stage I-II primary breast tumors and 95 autologous lymph node metastases for COX2, PGE_2S, EGFR, hormones receptor, HER2, p53, Ki67, Fas and FasL expression demonstrate that tumor size, nodal status, COX2, PGE_2S, Ki67, Fas and FasL expression were significant predictors of DFS while OS was significantly influenced by tumor size, nodal status, COX2, p53, Ki67, HER2, Fas and FasL expression. Besides a marked increase (11.4%) in COX2 expression in metastases versus primary tumors, significant correlation between COX2 expression and nodal status was found in our investigations. This finding may be explained with the data obtained by a study on murine model of metastatic breast cancer, where in contrast to the uniform in vitro COX2 expression, only tumors resulting from the transplantation of metastatic cell lines expressed COX2 in vivo suggesting that

i) in the tumor milieu, COX2 expression may be regulated differently in non-metastatic versus metastatic lesions (30) and that

ii) constitutive expression of COX2 may be required to maintain the altered phenotype of increased invasiveness (137).

Our samples showed a significant correlation between COX2 and PGE_2S expression in breast tumors. This finding is not unexpected, since human breast cancers were shown by others to contain high levels of PGE_2 provided by the breast fibroblasts under the influence of inflammatory mediators (138), and the ability of breast tumors to produce PGE_2 is also related to high COX2 expression and metastatic potential (138). PGE_2S was also related to ER expression in our samples. These results can be explained by the findings that the aromatase enzyme complex, which catalyzes estrogen biosynthesis, is regulated by PGE_2 via four transmembrane receptors (139) and via induction of interleukin-6 (140), thus providing the increased estrogen level fundamental to hormone-dependent growth of breast cancer.

In our series of samples we also found that COX2 was significantly related to Ki67 expression. Ki67 is a widely accepted marker of proliferation (141, 142), thus, in accordance with other authors (143, 144), relationship between Ki67 and other markers of unfavorable

outcome, such as COX2, may be based on the worse prognosis related to poorly differentiated and intensively proliferating cancers.

p53 expression alone significantly influenced only OS but not DFS, while when considering concomitant COX2 and p53 expression, this combination had a significant impact both on DFS and OS among our samples. Traditionally, p53 overexpression by immunohistochemistry is thought to represent *TP53* mutation, however, high concordance of increased p53 protein is only seen with missense mutations, which result in protein that is resistant to degradation and has longer half-life than the wild-type (wt) counterpart (145). Furthermore, our present data imply that breast tumors overexpressing p53 and COX2 have a significantly poorer DFS ($p=0.003$) and OS ($p=0.02$) than those with normal low pattern of p53 expression and no expressed COX2. Because the COX2 gene has been shown to be induced in p53 defective cells and down-regulated by wt p53 (146), there may exist a direct link between a defective p53 pathway and elevated levels of COX2 expression in cancer cells. Though we did not investigate that the expressed p53 protein in our samples were wt or mutant, our results indicate that the p53 protein expressed in the breast tumor samples may be at least partly defected mutant p53 protein thus failing to repress COX2 expression.

In contrast to the model system, COX2 expression was not associated to either EGFR or HER2 expression. Possible explanations, as offered by other authors (24, 147-151), are as follows:

(i) Tumor tissue specimens represent a different environment from those in *in vitro* models. The production of growth factors as EGFR as well as COX2 synthesis by endothelial and stromal cells contribute to realization of a regulatory microenvironment which might not fit the straight biochemical relationships found in cell culture models (147).

(ii) The association between high EGFR expression and poor response to chemotherapy was reported in studies utilizing a radioligand assay for EGFR determination (148, 149), while no association between EGFR and response to chemotherapy or clinical outcome was reported in cases of immunohistochemical assessment of EGFR expression (150, 151). This suggests that the methodological approach could heavily affect the evaluation of the prognostic value of the marker.

(iii) COX2 expression is regulated by other signaling pathways in addition to those promoted by the erb-B family members. In this context it is worth noting that inhibition of HER2/HER3 complex formation in colon cancer cells failed to completely inhibit COX2 protein expression (24).

We also demonstrated that in accordance with a cell culture model system (28) and another clinical study (152), EGFR and HER2 are associated one another. Furthermore, EGFR correlated with ER and PgR expression, which is in accordance with other findings (152). These results are not unexpected since it is postulated that estrogen or estrogen-regulated proteins are involved in the regulation of EGFR mRNA and protein (28), and that expression of ER and EGFR is required for controlling tumor proliferative activity in vitro (153).

ER was related to p53 expression. Since ER has been shown physically associate with the amino terminus of p53 to form complexes and protect p53 from degradation, it is highly probable that ER signaling results in the up-regulation of p53 expression to mediate G_1 cell cycle arrest (reviewed in 154). As mentioned above, in presence of non-functional (mutant) p53, despite of p53 overexpression, cell cycle has already broken loose from under control. Relationship between ER and Ki67 expression may be explained by the finding that activated ER can activate the mitogen activated protein kinase pathway (MAPK) thus resulting in increased proliferation (154).

Since ER is a key transcription factor for the activation of PgR and thus expression of PgR is highly regulated by ER expression (155), explanation of PgR expression related to Ki67, EGFR and p53 expression may be the same as provided for ER expression.

In accordance with other findings (143, 156), HER2 expression was significantly related to Ki67 expression and thus to high proliferation rate, which may be explained by an indirect relationship originating from both factors being well-known related to poor histological differentiation and increased malignancy of the disease.

p53 and FasL expression were significantly correlated, though no connection was observed between p53 and Fas. This finding further supports the hypothesis that p53 expressed in our tumor samples was non-functional, being able to induce FasL (157) but not Fas transcription (158), and unable to repress COX2 expression.

A significant relationship was found between HER2 and FasL expression. According to a spontaneous tumor model, concomitant HER2 and FasL expression is necessary for developing tumors being able to escape active specific immunotherapy by inducing apoptosis in tumor infiltrating T lymphocytes (159). These findings suggest that in patients with breast cancers overexpressing both biologic markers, the possibility of developing escape tumors must be considered when determining the most suitable therapy.

Fas and FasL expression was found to be significantly related to each other in our series of samples, with a consequence of more favorable prognosis than in patients with no Fas and FasL expression. This finding is not unexpected, since the Fas-FasL apoptotic system is

required for eliminating tumor cells, thus malignant cells with an intact apoptotic system are more probable to be eliminated than cells with a defective or completely missing Fas-FasL system (130, 133, 135).

Biologic markers used to determine prognosis of breast cancer patients are expressed as a net result of deregulated cell proliferation, thus concomitant evaluation of expression of these factors provide more accurate information on disease progression than independent investigation of the individual prognostic factors. Our results suggest that elevated expression of COX2 associates with poorer DFS and OS in breast cancers. Elevated PGE_2S activity in COX2 positive tumors derives from alteration in COX2 expression and results in ensured growth of hormone dependent tumor and in higher metastatic potential. Expression of COX2 associated with altered expression of tumor suppressor gene p53 and proliferative marker Ki67 may in part be responsible for induction of COX2 expression in breast cancer.

In conclusion, our data indicate that in high-risk breast cancer patients the immunohistochemical evaluation of COX2, together with PGE_2S, p53, Ki67, HER2, Fas and FasL, may be of clinical value in distinguishing different responses to adjuvant anthracycline-based chemotherapy. Furthermore, HER2 copy number determined by FISH not only in the tumor itself but also in the normal-appearing PTT may be of help in determining more accurate prognosis.

5. NEW STATEMENTS

1. In 5 cases out of the 10 HER2 positive peritumoral tissue samples (1cm) investigated showed HER2 amplification, thus supporting the hypothesis that the morphologically normal-appearing breast tissue already harbours molecular changes which may predict malignant transformation.
2. A gradient of expression was evident for FasL since the peritumoral tissues closest to invasive cancer showed an upregulated FasL ($p=0.05$) and this upregulation was lost in PTT farther from the invasive carcinoma ($p=0.04$ at 2 cm, $p=0.02$ at 3 cm). Thus, FasL, in combination with other biological parameters, may be a novel biomarker useful to identify patients at higher risk of developing recurrent breast cancer.
3. Tumor size, nodal status, COX2, PGE_2S, Ki67, Fas and FasL expression were significant predictors of DFS ($p=0.005$, $p<0.001$, $p=0.004$, $p=0.004$, $p=0.009$, $p<0.001$ and $p<0.001$ respectively) while OS was significantly influenced by tumor size, nodal status, COX2, p53, Ki67, HER2, Fas and FasL expression ($p=0.013$, $p=0.001$, $p=0.05$,

p=0.02, p=0.009, p=0.007, p<0.001 and p<0.001 respectively). Out of these investigated markers, COX2 and PGE2S may be used as new prognostic markers for breast cancer.

4. Concomitant overexpression of COX2 and p53 has significantly decreased 5 year disease free survival (p=0.003) and overall survival (p=0.02).
5. In high-risk breast cancer patients the immunohistochemical evaluation of COX2, together with PGE_2S, p53, Ki67, HER2, Fas and FasL, may be of clinical value in distinguishing different responses to adjuvant anthracycline-based chemotherapy.

6. ACKNOWLEDGEMENTS

Present work was supported by the Marie Curie Training Site Fellowship, European Program, HPMT-CT-200-00210.

I wish to thank Gyögy Kosztolányi MD, PhD, DSc and Béla Melegh MD, PhD, DSc for their guidance and mentorship at the beginning of my scientific work, Marcella Mottolese PhD for the excellent pathological classification of specimens and the professional guidance throughout the above project and Professor Pier Giorgio Natali MD for the possibility to work under his direction.

Furthermore, I'm grateful to István Ember MD, PhD, DSc who has made it possible for me to carry out this project and who always supported my scientific carreer and to all the members of the Department of Public Health and Preventive Medicine (University of Pécs, Faculty of Medicine) for their help, kindness and support.

I wish to express my gratitude to Patrizia Scordati and Valentina Zerbini for the excellent technical assistance.

At last but not at least, this work would have never been realized without the support and help of my family.

7. REFERENCES

1. Lundin C, Mertens F: Cytogenetics of benign breast lesions. Breast Canc Res Treat 51:1-15, 1998
2. Botti C, Pescatore B, Mottolese M, Sciarretta F, Greco C, Di Filippo F, Gandolfo GM, Cavaliere F, Bovani R, Varanese A, Cianciulli AM: Incidence of chromosomes 1 and 17 aneusomy in breast cancer and adjacent tissue: an interphase cytogenetic study. J Am Coll Surg 190(5):1-10, 2000
3. Crawford L, Pim D, Lamb P: The cellular protein p53 in human tumors. Mol Biol Med 2:261-267, 1984
4. Koutselini H, Malliri A, Field JK, Spandidos DA: p53 expression in cytologic specimens from benign and malignant breast lesions. Anticancer Res 11:1415-1420, 1991
5. Millikan R, Hulka B, Thor A, Zhang Y, Edgerton S, Zhang X, Poi H, He M, Wold L, Melton J, Ballard D, Conway K, Liu ET: p53 mutations in benign breast tissue. J Clin Oncology 13:2293-2300, 1995
6. Rohan TE, Hartwick W, Miller AB, Kandel RA: Immunohistochemical detection of c-erbB-2 and p53 in benign breast disease and breast cancer risk. J Natl Canc Inst 90(17):1262-1269, 1998
7. Schimmelpenning H, Eriksson ET, Pallis L, Skoog L, Cedermark B, Auer GU: Immunohistochemical c-erbB-2 protooncogene expression and nuclear DNA content in human mammary carcinoma *in situ*. Am J Clin Pathol 97(5 Suppl 1):S48-S52, 1992
8. Nagata S: Apoptosis by death factor. Cell 88:355-365, 1997
9. Stark A, Hulka BS, Joens S, Novotny D, Thor AD, Wold LE, Schell MJ, Melton LJ, Liu ET, Conway K: Her-2/neu amplification in benign breast disease and the risk of subsequent breast cancer. J Clin Oncol 18(2):267-274, 2000
10. Xu R, Perle MA, Inghirami G, Chan W, Delgado Y, Feiner H: Amplification of Her-2/*neu* gene in Her-2/neu-overexpressing and -nonexpressing breast carcinomas and their synchronous benign, premalignant, and metastatic lesions detected by FISH in archival material. Mod Pathol 15(2):116-124, 2002
11. Mottolese M, Buglioni S, Bracalenti C, Cardarelli MA, Ciabocco L, Giannarelli D, Botti C, Natali PG, Concetti A, Venanzi FM: Prognostic relevance of altered Fas (CD-95)-system in human breast cancer. Int J Cancer (Pred Oncol) 89:127-132, 2000

12. Herrnring C, Reimer T, Jeschke U, Makovitzky J, Kruger K, Gerber B, Kabelitz D, Friese K: Expression of the apoptosis-inducing ligands FasL and TRAIL in malignant and benign human breast tumors. Histochem Cell Biol 113(3):189-194, 2000
13. Mullauer L, Mosberger I, Grusch M, Rudas M, Chott A: Fas ligand is expressed in normal breast epithelial cells and is frequently up-regulated in breast cancer. J Pathol 190(1):468-470, 2000
14. Thun MJ, Namboodiri MM, Calle EE, Flanders WD, Heath CW Jr: Aspirin use and risk of fatal cancer. Cancer Res 53:1322-7, 1993
15. Gupta RA, DuBois RN: Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nature Reviews / Cancer 1:11-21, 2001
16. Dannenberg AJ, Altorki NK, O'Boyle J, Dang C, Howe LR, Weksler BB, Subbaramaiah K: Cyclo-oxygenase-2: a pharmacological target for the prevention of cancer. Lancet Onc 2:544-551, 2001
17. Howe LR, Subbaramiah K, Chung WJ, Dannenberg AJ, Brown AMC: Transcriptional activation of cyclooxygenase-2 in Wnt-1- transformed mouse mammary epithelial cells. Cancer Res 59:1572-1577, 1999
18. Harris RE, Namboodiri KK, Farrar WB: Nonsteroidal antiinflammatory drugs and breast cancer. Epidemiology 7:203-5, 1996
19. Harris RE, Namboodir, KK, Farrar WB: Epidemiological study of nonsteroidal antiinflammatory drugs and breast cancer. Oncol Rep 2:591-592, 1995
20. Hwang D, Scollard D, Byrne J, Levine E: Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer. J Natl Canc Inst 90(6):455-460, 1998
21. Taketo MM: Cyclooxygenase-2 inhibitors in tumorigenesis (part I). J Natl Cancer Inst 90:1529-36, 1998
22. Taketo MM: Cyclooxygenase-2 inhibitors in tumorigenesis (part II). J Natl Cancer Inst 90:1609-20, 1998
23. Subbaramiah K, Telang N, Ramonetti JT, Araki R, DeVito B, Weksler BB, Dannenberg AJ: Transcription of cyclooxygenase-2 is enhanced in transformed mammary epithelial cells. Cancer Res 56:4424-4429, 1996
24. Vadlamudi R, Mandal M, Adam L, Steinbach G, Mendelsohn J, Kumar R: Regulation of cyclooxygenase-2 pathway by HER2 receptor. Oncogene 18(2):305-314, 1999
25. Howe LR, Subbaramiah K, Brown AMC, Dannenberg AJ: Cyclooxygenase-2: a target for the prevention and treatment of breast cancer. Endocr Relat Cancer 8:97-114, 2001

26. Kundu N, Yang Q, Dorsey R, Fulton AM: Increased cyclooxygenase-2 (COX-2) expression and activity in a murine model of metastatic breast cancer. Int J Cancer 94:681-686, 2001

27. Liu CH, Chang S-H, Narko K, Trifan OC, Wu M-T, Smith E, Haudenschild C, Lane TF, Hla T: Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice. J Biol Chem 276(21):18563-18569, 2001

28. Gilhooly EM, Rose DP: The association between a mutated ras gene and cyclooxygenase-2 expression in human breast cancer cell lines. Int J Oncol 15(2):267-270, 1999

29. Sotiriou C, Lacroix M, Lagneaux L, Berchem G, Body JJ: The aspirin metabolite salicylate inhibits breast cancer cells growth and their synthesis of the osteolytic cytokines interleukins-6 and –11. Anticancer Res 19(4B):2997-3006, 1999

30. Soslow RA, Dannenberg AJ, Rush D, Woerner BM, Khan KN, Masferrer J, Koki AT: Cox-2 is expressed in human pulmonary, colonic, and mammary tumors. Cancer 89(12):2637-2645, 2000

31. Liu XH, Rose DP: Differential expression and regulation of cyclooxygenase-1 and –2 in two human breast cancer cell lines. Cancer Res 56(22):5125-5127, 1996

32. Egan KM, Stampfer MJ, Giovannucci E, Rosner BA, Colditz GA: Prospective study of regular aspirin use and the risk of breast cancer. J Natl Cancer Inst 88:988-993, 1996

33. Friedman GD, Ury HK: Initial screening for carcinogenicity of commonly used drugs. J Natl Cancer Inst 65:723-733, 1980

34. Pai R, Soreghan B, Szabo IL, Pavelka M, Baatar D, Tarnawski AS: Prostaglandin E_2 transactivates EGF receptor: A novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. Nature Med 8(3):289-293, 2002

35. Cotran RS, Kumar V, Robbins SL. The Breast. In: Cotran R S, Kumar V, Robbins S L. Robins Pathologic Basis of Disease. 4th ed. Philadelphia, London, Toronto, Montreal, Sydney, Tokyo: W.B. Saunders Company, 1989. p. 1181-204.

36. Sadler TW. Langman's Medical Embryology. Fifth ed. Baltimore, London, Sydney: Williams & Wilkins, 1985. p. 331-333.

37. Sperof L, Glass RH, Kase NG. Clinical Gynecologic Endocrinology and Infertility. Baltimore: Wiliams & Wilkins; 1994. p. 547-5582.

38. Harper P. Ultrasound Mammography. Baltimore: University Park Press; 1985.

39. Bray F, Sankila R, Ferlay J, Parkin DM. Estimates of cancer incidence and mortality in Europe in 1995. Eur J Cancer 38:99-166, 2000

40. Levi F, Lucchini F, Negri E, La Vecchia C. Worldwide patterns of cancer mortality, 1990-1994. Eur J Cancer Prev 8:381-400, 1999
41. Greenall M J. Cancer of the breast. In: Oxford textbook of Surgery, N York: Oxford University Press, 1994.
42. Donegan WL, Redlich PN. Breast cancer in men. Surg Clin North Am 76(2):343-363, 1996
43. Weiss S E, Tartter P I, Ahmed S, Brower S T, Brusco C, Bossolt K, Amberson J B, Bratton J. Ethnic Differences in Risk and Prognostic Factors for Breast Cancer. Cancer 76:268-274, 1995
44. Harris JR, Lippman ME, Morrow M, Hellman S. Disease of the Breast. Philadelphia - New York: Lippincott – Raven, 1996. p. 159-355.
45. Muszbek N, Koncz T, V Hajdú P, Ádány R. [Economic evaluation of population-based mass screening for the early detection of cancer: a systematic review.] Magy Onkol 46(2):119-129, 2002
46. Havasi V, Sándor J, Kiss I, Szűcs M, Brázay L, Ember I. [Mortality of breast cancer and frequency of mammography in Hungary.] Orv Hetil 142(50):2773-2778, 2002
47. Józan P. [Distress and reform in the development of epidemiology after World War II in Hungary.]. DSc thesis, 2004 December (Library of the national Academy of Sciences)
48. Boncz I, Sebestyén A, Gulácsi L, Pál M, Dózsa C. [Health economics analysis of breast cancer screening.] Magy Onkol 47(2):149-154, 2003
49. Péntek Z. [National and international experiences of mammographic breast screening.] LAM 12(4):232-234, 2002
50. Henderson I C, Harris J R, Kinne D W, Hellman S. Cancer of the Breast. In: DeVita VT, Hellman S, Rosenberg SA, editors. Cancer Principles & Practice of Oncology. Philadephia, Grand Rapids New York, St. Louis, San Francisco, London, Sydney, Tokyo: J. B. Lippincott Company, 1989. p. 1197-268.
51. Anelli A, Anelli TFM, Youngson B, Rosen P, Borgen PI. Mutations of the p53 Gene in Male Breast Cancer. Cancer 75:2233-2238, 1995
52. Thompson WD. Genetic Epidemiology of Breast Cancer. Cancer 74(Suppl 1):279-287, 1994
53. Kinoshita T, Ueda M, Enomoto K, Ikeda T, Kikuchi K, Ishii S, Kitajima M. Comparation of p53 Gene Abnormalities in Bilateral and Unilateral Breast Cancer. Cancer 76:2504-2509, 1995

54. FitzGerald MG, MacDonald DJ, Krainer M, Hoover I, O'Neil E, Unsal H, Arrieto SS, Finkelstein DM, Beer-Romero P, Englert C. Germline BRCA1 mutations in Jewish and non-Jewish woman with early onset breast cancer. N Engl J Med 334:3:143-149, 1996

55. Hayes F. When Is a Tumor Marker Ready for Prime Time? In: Perry M C, editor. American Society of Clinical Oncology. Washington, Boston, San Antonio: American Society of Clinical Oncology, 1997. p. 206-212.

56. Garber J. Breast Cancer Markers: Genetic Markers of Breast Cancer Predisposition. In: Perry M C, editor. Washington, Boston, San Antonio: American Society of Clinical Oncology, 1997. p. 213-216.

57. Ravdin P M. Prognostic Factors in Breast Cancer. In: Perry M C, editor. Washington, Boston, San Antonio: American Society of Clinical Oncology, 1997. p. 217-227.

58. Hayes D. Serum (Circulating) Tumor Markers for Breast Cancer. In: Perry M C, editor. Washington, Boston, San Antonio: American Society of Clinical Oncology, 1997. p. 228-233.

59. Cole DE, Gallinger S, McCready DR, Rosen B, Engel J, Malkin D. Genetic counselling and testing for suspectibility to breast, ovarian and colon cancer: where are we today? Can Med Assoc J 154:2: 149-155, 1996

60. Lancaster JM, Wiseman RW, Berchuck A. An inevitable dilemma: prenatal testing for mutations in the BRCA1 breast-ovarian cancer susceptibility gene. Obstet Gynecol 87(2): 306-309, 1996

61. Radford DM, Zehnbauer BA. Inherited breast cancer. Surg Clin North Am 76(2):205-220, 1996

62. Langston AA, Malone KE,Thompson JD,Daling JR, Ostrander EA. BRCA1 mutations in a population - based sample of young women with breast cancer. N Engl J Med 334(3):137-142, 1996

63. Kuller LH. The etiology of breast cancer from epidemiology to prevention. Public Health Rev 23(2):157-213, 1995

64. Nason FG, Nelson BE. Estrogen and progesteron in breast and gynecologic cancers. Etiology, therapeutic role, and hormone replacement. Obstet Gynecol Clin North Am 21:2:245-270, 1994

65. Bagga D, Ashley J M, Geffrey SP, Wang HJ, Barnard RJ, Korenman S, Heber D. Effects of a Very Low Fat, High Fiber Diet on Serum Hormones and Menstrual Function. Cancer 76:2491-2496, 1995

66. Kumar N B, Lyman G H, Allen K, Cox C E, Schapira D V. Timing of Weight Gain and Breast Cancer Risk. Cancer 76:243-249, 1995

67. Frisch R, Gotz-Welbergen A, McArthur J. Delayed menarche and amenorrhea of college athlethes in relatio to age at onset of training. JAMA 246:1559, 1981

68. Rookus M A, van Leeuwen F E. Oral contraceptives and risk of breast cancer in women aged 20-54 years. The Lancet 344:844-851, 1994

69. Colditz G A, Hanminson S E, Hunter D J, Willett W C, Manson J E, Stampfer M J, Hennekens C, Rosner B, Speizer F E. The use of Estrogens and Progestins and the Risk of Breast Cancer in Postmenopausal Women. N Engl J Med 332:1589-1593, 1995

70. Davidson N E. Hormone - replacement therapy - Breast versus Heart versus Bone. N Engl J Med 332:1638-1639, 1995

71. Mann R D, editor. Hormone Replacement Therapy and Breast Cancer Risk. 1st ed. Lancs, New Jersey:The Parthenon Publishing Group, 1992. p. 169-170.

72. Newell GR. Advising Patients About Benefits and Cancer Risks of Taking Hormones. Primary Care & Cancer 11(6):23-25, 1991

73. McMhann CF. Pitfalls and Problems in Breast Palpitation. Primary Care & Cancer 11)6):15-21, 1991

74. Speroff L. Postmenopausal hormone therapy and breast cancer. Obstet Gynecol 87(Suppl2):44-54, 1996

75. Page DL. The woman at high risk for breast cancer. Importance of hyperplasia. Surg Clin North Am 76(2):221-230, 1996

76. Beral V. Breast cancer mortality in England and Wales, 1950-93. Lancet 345:1642-1643, 1995

77. Another look at the pill and breast cancer (editorial). Lancet 2:985-987, 1985

78. McPherson K, Neil A, Vessey M. Oral contraceptives and breast cancer. (Letter) Lancet 2:1414, 1983

79. Siiteri P, Hammond G, Nishker J. Increased availability of serum estrogens in breast cancer: a new hypothesis. In: Pike M, Siiteri P, Welsh C. Hormones and breast cancer. NY: Cold Spring Harbor Laboratories, 1981.

80. de Waard F, Cornelis J, Aoki K. Breast cancer incidence accoding to weight and height in two cities of the Netherlands and in Aichi Prefecture, Japan. Cancer 40:1269, 1977

81. Schapira D V, Clarc R A, Wolff P A, Jarrett A R, Kumar N B, Aziz N M. Visceral Obesity and Breast Cancer Risk. Cancer 74:632-639, 1994

82. Hunter DJ, Spiegelman D, Adami HO, Beeson L, Brandt PA, Folsom AR, Fraser GE, Goldbohm RA, Graham S, Howe GR i sur. Cohort studies of fat intake and risk of breast cancer a pooled analysis. N Engl J Med 334(6):356-361, 1996

83. Vatten L J, Kvinnsland S. Cigarette Smoking and Risk of Breast Cancer: a Prospective Study of 24329 Norwegian Women. Eur J Cancer 26:830-833, 1990

84. Colditz G A. Epidemiology of Breast Cancer. Cancer 71:1480-1489, 1993

85. Henderson B I. Breast Cancer. In: Isselbacher K J, Braunwald E, Wilson J D, Martin J B, Fauci A S, Kasper D. Harrison's principles of internal medicine. New York: McGraw-Hill, 1994. p. 1840-1850.

86. Giuliano A E Breast Cancer In: Berek J S, Adashi E Y, Hillard P A. Novak's Gynecology. 12nd ed. Baltimore, London, Paris: Williams & Wilkins, 1996:1283-1302.

87. Petrinec Z, Huljev D. Selenium Levels in Blood Serum of Females with Breast Cancer. Libri Oncol 23:211-213, 1994

88. Hunter D J, Manson J E, Colditz G A. A prospective study of consumption of vitamins C, E and A and breast cancer risk. N Engl J Med 329:234, 1993

89. Martin-Moreno JM, Willett WC, Gorgojo L. Dietary fat, olive oil intake and breast cancer risk. Int J Cancer 58:774, 1994

90. Brinton A, Devesa SS. Environmental Factors. In: Harris JR, Lippman ME, Morrow M, Hellman S. Disease of the Breast. Philadelphia - New York: Lippincott – Raven, 1996. p. 159-168.

91. Ziegler RG, Hoover RN, Pike MC, Hildesheim A, Nomura AMY, West DW, Wu-Williams AH, Kolonel LN, Horn-Ross PL, Rosenthal JF, Hyer MB. Migration Patterns and Breast Cancer Risk in Asian - American Women. J Natl Cancer Inst 85:1819-1827, 1993

92. Halbreich U, Shen J, Panaro V. Are chronic psychiatric patients at increased risk for developing breast cancer? Am J Psychiatry 153(4):559-560, 1996

93. Rosen PP, Oberman HA. Atlas of Tumor Pathology. Tumors of the Mammary Gland.Washington: Armed Forces Institute of Pathology, 1993. p. 7-10.

94. McDivitt RD, Stewart FW, Berg JW: Tumors of the Breast. Washington: Armed Forces Institute of Pathology, 1968.

95. World Health Organization: Histologic typing of breast tumors. Washington: The Institute, 1982.

96. Sharkey FE, Allred DC, Valente PT. Breast In: Kissane JM, editor. Anderson's Pathology. Ninth Edition. St. Louis, Baltimore, Philadelphia, Toronto, Mosby, 1990. p. 2354-2385.

97. Netter FH. The CIBA collection of medical illustrations. Vol 2. Reproductive system. New York, CIBA, 1970, pp 243-266

98. Porrath S. A Multimodality Approach to Breast Imaging. 1th ed. Rockville, Maryland: Aspen, 1986, p 80.

99. Ezo MG, Hagen Ansert SL. Breast. In: Hagen - Ansert SL. Textbook of diagnostic ultrasonography. Third ed. St. Louis, Baltimore, Philadelphia, Toronto, Mosby, 1989, pp. 338-354.

100. Wilson J F. The Breast. In:Moss W T, Cox J D, editors. Radiation Oncology. 6 th Edition. St. Louis, Baltimore, Philadelphia, Toronto, Mosby, 1989, pp. 312-337.

101. Mansour EG, Ravdin PM, Dressler L. Prognostic Factors in Early Breast Carcinoma. Cancer 74(Suppl 1):381-400, 1994

102. Nair K, Sankaranarayanan R, Nair S, Amma NS Varghese C, Padmakumari G, Cherian T. Overall Survival from Breast Cancer in Kerala, India, in Relation to Menstrual, Reproductive, and Clinical Factors. Cancer 71:1791-1796, 1993

103. Clayton F, Hopkins CL. Pathologic Correlates of Prognosis in Lymph Node - Positive Breast Carcinomas. Cancer 71:1780-1790, 1993

104. Querzoli P, Ferretti S, Albonico G, Magrio E, Scapoli D, Indelli M, Nenci I. Application of Quantitative Analysis to Biologic Profile Evaluation in Breast Cancer. Cancer 76:2510-2517, 1995

105. American Joint Committee on Cancer. Manual for Staging of Cancer. 3 th Edition. Philadelphia, London, Mexico, New York, St. Louis, Sao Paulo, Sydney: J. B. Lippincott Company, 1988. pp. 145-150.

106. Wood WC. Progress from Clinical Trials on Breast Cancer. Cancer 74:2606-2609, 1994

107. Byrne C, Smart C R, Chu K C, Hartmann H. Survival Advantage Differences by Age. Cancer 74(Suppl 1):301-310, 1994

108. Jancar B. Breast cancer screening. Radiol Oncol 27:232-235, 1993

109. Eddy DM, Hasselblad V, McGivney W. The value of mammography screening in women under age 50 years. JAMA 259:1512-1519, 1988

110. Mettlin C. The Relationship of Breast Cancer Epidemiology to Screening Recommendations. Cancer 74(Suppl 1):228-230, 1994

111. Digiesi G, Giacomini P, Fraioli R, Mariani M, Nicotra MR, Segatto O, Natali PG: Production and characterization of murine mAbs to the extracellular domain of human neu oncogene product GP185HER2. Hybridoma 11(4):519-527, 1992

112. Ristimaki, A., Sivula, A., Lundin, J., Lundin, M., Salminen, T., Haglund, C., Joenssu, H., Isola, J.: Prognostic significance of elevated cyclooxygenase-2 expression in breast cancer. Cancer Res 62:632-635, 2002

113. Dairkee SH, Smith HS: Genetic analysis of breast cancer progression. J Mamm Gland Biol Neoplasia 1:139-152, 1996

114. Hassan IH, Walker RA: Decreased apoptosis in non-involved tissue from cancer-containing breast. J Pathol 184:258-264, 1998

115. Deng G, Lu Y, Zlotnikov G, Thor AD, Smith HS: Loss of heterozygosity in normal tissue adjacent to breast carcinomas. Science 274:2057-2059, 1996

116. Li D, Wang M, Dhingra K, Hittelman WN: Aromatic adducts in adjacent tissues of breast cancer patients: clues to breast cancer ethiology. Cancer Res 56:287-293, 1996

117. Mommers ECM, van Diest PJ, Leonhart AM, Maijer CJLM, Baak JPA: Expression of proliferation and apoptosis-realetd proteins in usual ductal hyperplasia of the breats. Hum Pathol 29(12):1539-1545, 1998

118. Ratcliffe N, Wells W, Wheeler K, Memoli V : The combination of *i*n *s*itu *h*ybridisation and immunohistochemical analysis: an evaluation of *Her2/neu* expression in paraffin-embedded breast carcinomas and adjacent normal-appearing breast epithelium. Mod Pathol 0(12):1247-1252, 1997

119. Starcevic SL, Elferink C, Novak RF: Progressive resistance to apoptosis in a cell lineage model of human proliferative breast cancer. J Natl Cancer Inst 16(93):776-782, 2001

120. Hung MC, Lau YK: Basic science of HER-2/neu: a review. Semin Oncol 26:51-59, 1999

121. Roetger A, Merschjann A, Dittmar T, Jackisch C, Barnekow A, Brandt B: Selection of potentially metastatic subpopulations expression c-ErbB-2 from breast cancer tissue by use of an extravasation model. Am J Pathol 153(6):1797-1806, 1998

122. Van Parijs L, Abbas AK: Homeostasis and self-tolerance in the immune system: turning lymphocytes off. Science 280:243-248, 1998

123. Wong B, Choi Y: Pathways leading to death in T cells. Curr Opin Immunol 9:358-364, 1997

124. O'Connell J, O'Sullivan GC, Collins JK, Shanahan F: the Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J Exp Med 184:1075-1082, 1996

125. Jacobs TW, Gown AM, Yaziji H, Barmes MJ, Schnitt SJ. Specificity of hercepTest in determining Her2/neu status of breast cancer using the United States Food and Drug Administration approved scoring system. J Clin Oncol 17(7):1983-1987, 1999

126. Fabian CJ, Kimler BF, Zalles CM, Klemp JR, Kamel S, Zeiger S, Mayo MS: Short-term breast cancer prediction by random periareolar fine-needle aspiration cytology and the Gail risk model. J Natl Cancer Inst 92:1217-1227, 2000

127. Allred DC, Hilsenbeek SG. Biomarkers in benign breast disease: risk factors for breast cancer. J Natl Cancer Inst 90(17):1247-1248, 1998

128. Song J, Sapi E, Brown W, Nilsen J, Tartaro K, Kacinski BM, Craft J, Naftolin F, Mor G: Role of Fas and Fas ligand during mammary gland remodeling. J Clin Invest 106:1209-1220, 2000

129. Reimer T, Herrnring C, Koczan D, Richter D, Gerber B, Kabelitz D, Friese K, Tjiesen HJ: FasL:Fas ration - a prognostic factor in breast carcinomas. Cancer Res 60:822-828, 2000

130. Zeytun A, Hassuneh M, Nagarkatti M, Nagarkatti PS: Fas-Fas ligand-based interactions between tumor cells and tumor-specific cytotoxic T lymphocytes: a lethal two-way street. Blood 90(5):1952-1959, 1997

131. Trapani JA: Tumor-mediated apoptosis of cancer-specific T lymphocytes - Reversing the "kiss of death"? Cancer Cell 2(3):169-171, 2002

132. Pinkoski MJ, Green DR: Murder by proxy. Nat Immunol 1(6):461-462, 2000

133. Owen-Schaub LB. Fas function and tumor progression: use it and lose it. Cancer Cell 2(2):95-96, 2002

134. Pinkoski MJ, Droin NM, Lin T, Genestier L, Ferguson TA, Green DR: Nonlymphoid Fas ligand in peptide-induced peripheral lymphocyte deletion. Proc Natl Acad Sci USA 99(25):16174-16179, 2002

135. Gutierrez LS, Eliza M, Niven-Fairchild T, Naftolin F, Mor G: The Fas/Fas-ligand system: a mechanism for immune evasion in human breast carcinomas. Breast Cancer Res Treat 54(3):245-253, 1999

136. Céfai D, Schwaninger R, Balli M, Brunner T, Gimmi CD: Functional characterization of Fas ligand on tumor cells escaping active specific immunotherapy. Cell Death Diff 8:687-695, 2001

137. Tsujii, M., Kawano, S., DuBois, R. N.: Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. Proc Natl Acad Sci USA, 94:3336-3340, 1997

138. Ferrandina, G., Lauriola, L., Distefano, M. G., Zannoni, G. F., Gessi, M., Legge, F., Maggioano, N., Mancuso, S., Capelli, A., Scambia, G., Ranelletti, F. O.: Increased cyclooxygenase-2 expression is associated with chemotherapy resistance and poor survival in cervical cancer patients. J Clin Oncol 20(4):973-981, 2002

139. Richards, J. A., Brueggemeier, R. W.: Prostaglandin E2 regulates aromatase activity and expression in human adipose stromal cells via two distinct receptor subtypes. J Clin Endocrinol Metab 88(6):2810-2816, 2003

140. Singh, A., Purohit, A., Ghilchik, M. W., Reed, M. J.: the regulation of aromates activity in breast fibroblasts: the role of interleukin-6 and prostaglandin E_2. Endocr Relat Cancer 6:139-147, 1999

141. Chang J, Powles TJ, Allred DC, Ashley SE, Clark GM, Makris A, Assersohn L, Gregory RK, Osborne CK, Dowsett M: Biologic markers as predictors of clinical outcome from systemic therapy for primary operable breast cancer. J Clin Oncol 17(10):3058-3063, 1999

142. Jones S, Clark G, Koleszar S, Ethington G, Mennel R, Paulson S, Brooks B, Kerr R, Denham C, Savin M, White C, Blum J, Kirby R, Stone M, Pippen J, Kitchens L, George T, Cooper B, Peters G, Knox S, Grant M, Cheek H, Jones R, Kuhn J, Lieberman Z, Savino D, Rietz C: Low proliferative rate of invasive node-negative breast cancer predicts for a favorable outcome: a prospective evaluation of 669 patients. Clin Breast Cancer 1(4):310-317, 2001

143. Rudas M, Neumayer R, Gnant MFX, Mittelbock M, Jakesz R, Reiner A: p53 Protein Expression, Cell Proliferation and Steroid Hormone Receptors in Ductal and Lobular *In Situ* Carcinomas of the Breast. Eur J Cancer 33(1):39-44, 1997

144. Midulla C, De Iorio P, Nagar C, Pisani T, Cenci M, Valli C, Nofroni I, Vecchione A: Immunohistochemical expression of p53, nm23-HI, Ki67, and DNA ploidy: correlation with lymph node status and other clinical pathologic parameters in breast cancer. Anticancer Res 19(5B):4033-4037, 1999

145. Erkinheimo T-L, Lassus H, Finne P, van Rees BP, Leminen A, Ylikorkala O, Haglund C, Butzow R, Ristimaki A: Elevated Cyclooxygenase-2 expression Is associated with Altered Expression of p53 and SMAD4, Amplification of HER-2/neu, and Poor Outcome in Serous Ovarian carcinoma. Clin Cancer Res 10:538-545, 2004

146. Subbaramaiah, K., Altorki, N., Chung, W. J., Mestre, J. R., Sampat, A., Dannenberg, A. J.: Inhibition of cyclooxygenase-2 gene expression by p53. J Biol Chem 274(16):10911-10915, 1999

147. Ferrandina, G., Ranelletti, F. O., Lauriola, L., Fanfani, F., Legge, F., Mottolese, M., Nicotra, M. R., Natali, P. G., Zakut, V. H., Scambia, G.: Cyclooxygenase-2 (COX-2), Epidermal Growth Factor Receptor (EGFR) and Her-2/neu expression in ovarian cancer. Gynecol Oncol 85:305-310, 2002

148. Scambia, G., Benedetti-Panici, P., Ferrandina, G., Distefano, M., Salerno, G., Romanini, M. E., Fagotti, A., Mancuso, S.: Epidermal growth factor, oestrogen and progesterone receptor expression in primary ovarian cancer: correlation with clinical outcome and response to chemotherapy. Br J Cancer 72(2):361-366, 1995

149. Fischer-Colbrie, J., Witt, A., Heinzl, H., Speiser, P., Czerwenka, K., Sevelda, P., Zeillinger, R.: EGFR and steroid receptors in ovarian carcinoma: comparison with prognostic parameters and outcome of patients. Anticancer Res 17(1B):613-619, 1997

150. Baekelandt, M., Kristensen, G. B., Trope, C. G., Nesland, J. M., Holm, R.: Epidermal growth factor receptor expression has no independent prognostic significance in advanced ovarian cancer. Anticancer Res 19(5C):4469-4474, 1999

151. Goff, B. A., Ries, J. A., Els, L. P., Coltrera, M. D., Gown, A. M.: Immunophenotype of ovarian cancer as predictor of clinical outcome: evaluation at primary surgery and second-look procedure. Gynecol Oncol 70(3):378-385, 1998

152. Hainsworth, P.J., Henderson, M. A., Stillwell, R. G., Bennett, R. C.: Comparison of EGFR, c-erbB-2 product and ras p21 immunohistochemistry as prognostic markers in primary breast cancer. Eur J Surg Oncol 17(1):9-15, 1991

153. Saxena, S., Jain, A. K., Pandey, K. K., Dewan, A. K.: Role of steroide hormone and growth factor receptors and proto-oncogenes in the behaviour of human mammary epithelial cancer cells in vitro. Pathobiology 65(2):75-82, 1997

154. Keen JC, Davidson NE: The Biology of Breast Carcinoma. Cancer. Cancer 97(Suppl 3):825-833, 2003

155. Graham JD, Roman SD, McGowan E, Sutherland RL, Clarke CL: Preferential stimulation of human progesterone receptor B expression by estrogen in T-47D human breast cancer cells. J Biol Chem 270:30693-30700, 1995

156. Lebeau A, Unholzer A, Amann G, Kronawitter M, Bauerfeind I, Sendelhofert A, Iff A, Lohrs U: EGFR, HER-2/neu, cyclin D1, p21 and p53 in correlation to cell proliferation

and steroid hormone receptor status in ductal carcinoma *in situ* of the breast. Breast Cancer Res Treat 79:187-198, 2003

157. Muller M, Wilder S, Bannasch D, Israeli D, Lehlbach K, Li-Weber M, Friedman SL, Galle PR, Stremmel W, Oren M, Krammer PH: p53 Activates theCD95 (APO-1/Fas) Gene in Response to DNA Damage by Anticancer Drugs. J Exp Med 188(11):2033-2045, 1998
158. Li Y, Raffo AJ, Drew L, Mao Y, Tran A, Petrylak DP, Fine RL: Fas-Mediated Apoptosis is Deependent on Wild-Type p53 Status in Human Cancer Cells Expressing a Temperature-Sensitive p53 Mutant Alanine-143[1]. Cancer Res 63:1527-1533, 2003
159. Céfai D, Favre L, Wattendorf E, Marti A, Jaggi R, Gimmi CD: Role of Fas ligand in promoting escape from immune rejection in a spontaneous tumor model. Int J Cancer 91(4):529-537, 2001

PUBLICATIONS

Papers related to the thesis:

1. Phenotypic changes of p53, HER2, and Fas system in multiple normal tissues surrounding breast cancer

   Mottolese M, **Nádasi E,** Botti C, Cianciulli AM, Merola R, Buglioni S, Benevolo M, Giannarelli D, Marandino F, Del Monte G, Venturo I, Natali PG

   J Cell Physiol (in press) **IF: 5.463**
2. Prognostic factors in breast cancer patients

   Nádasi E, Sándor J, Mottolese M, Ember I

   Anticancer Res (accepted for publication) **IF: 1.347**
3. [Role of interactions between oncogenes and suppressor genes in the pathogenesis of breast cancer]

   Nádasi E, Sándor J, Ember I

   Magy Onkol (submitted)

Papers not related to the thesis

1. Detection of Leu40Arg variant of platelet glycoprotein IIb/IIIa receptor in subjects with thrombotic diseases

   Nádasi E, Bene J, Havasi V, Komlósi K, Talián G, Melegh G, Papp E, Gasztonyi B, Szolnoki Z, Mózsik G, Tóth K, Sándor J, Melegh B, Wittmann I

   Thromb Res (in press) **IF: 1.710**

2. Carcinogenic potential of trans-2-hexenal is based on epigenetic effect
   Nádasi E, Varjas T, Pajor L, Ember I
   In Vivo (in press) **IF: 0.753**
3. Early effects of Transplatin on oncogene activation *in vivo*
   Németh Á, **Nádasi E,** Beró A, Olasz L, Nyárádi Z, Ember Á, Kvarda A, Bujdosó L, Arany I, Csejtey A, Faluhelyi Z, Ember I
   Anticancer Res (in press) **IF: 1.347**
4. [Mitochondrial DNA deletions in newborn brain samples]
   Nádasi E, Melegh B, Seress L, Kosztolányi Gy
   Orv Hetil 145(25):1321-1325, 2004
4. Early effects of different cytostatic protocols for head and neck cancer on oncogene activation in animal experiments
   Németh Á, **Nádasi E,** Gyöngyi Z, Olasz L, Nyárádi Z, Ember Á, Kvarda A, Bujdosó L, Arany I, Kiss I, Ember I
   Anticancer Res 23(6C):4831-4836, 2003 **IF: 1.347**
5. Mitochondrial DNA^{4977} deletion in brain of newborns died after intensive care
   Nádasi E, Melegh B, SeressL, Kosztolányi G
   Acta Biol Hung 54(3-4):253-262, 2003 **IF: 0.309**
6. Congenital cataract as first symptom of a neuromuscular disease caused by a novel single large scale mitochondrial DNA deletion
   Bene J, **Nádasi E,** Kosztolányi G, Méhes K, Melegh B
   Eur J Hum Genet 11(5):375-379, 2003 **IF: 3.669**
7. Flow cytometric analysis of DMBA-induced early in vivo ras expression
   Gyöngyi Z, Grama L, **Nádasi E,** Sándor J, Németh Á, Varga C, Kiss I, Ember I
   In Vivo 16(5):323-326, 2002 **IF: 1.115**
8. Effect of cisplatin treatment on early activation of oncogenes in vivo
   Németh Á, Gyöngyi Z, **Nádasi E,** Ember Á, Olasz I, Nyárádi Z, Skapinyecz J, Ember I
   In Vivo 16(5):307-310, 2002 **IF: 1.115**
9. Long term effects of 1-nitropyrene on oncogene and tumor suppressor gene expression
   Gyöngyi Z, **Nádasi E,** Varga C, Kiss I, Ember I
   Anticancer Res 21(6A):3937-3940, 2001 **IF: 1.416**
5. [Molecular biology based method for screening for fragile X syndrome]
   Kovács E, Morava É, **Nádasi E,** Czakó M, Melegh B, Kosztolányi G
   Orv Hetil 139(52):3121-3123, 1998

6. [Diagnosing of Prader-Willi syndrome by cytogenetical and molecular genetical methods]
   Varjas T**, Nádasi E,** Kovács E, Molnár J, Melegh B, Kosztolányi G
   Orv Hetil 139(28):1685-1687, 1998
7. [Evaluation of CTG-triplet expansion in three generations of a family with dystrophical myotonia]
   Molnár J, Kis A, Melegh B, **Nádasi E,** Varjas T, Kovács E, Kosztolányi G
   Orv Hetil 139(18):1083-1085, 1998

Published abstracts related to the thesis

1. HER2, p53, Fas, FasL, COX2, PGE2S, EGFR expression in breast cancer and in normal peritumoral breast tissue: potential novel risk biomarkers
   Nádasi E, Sándor J, Mottolese M, Cianciulli AM, Natali PG, Ember I
   Anticancer Res 24(5D):3574, 2004 **IF: 1.347**
2. Immunohistochemical evaluation of HER2, p53, Fas and Fas ligand in breast cancer and multiple adjacent non-neoplastic breast tissues
   Nádasi E, Botti C, Natali PG, Buglioni S, Benevolo M, Venturo I, Del Monte G, Sciarretta F, Giannarelli D, Mottolese M
   Annals Oncol 13(Suppl 5):35-36, 2002 **IF: 3.153**

Published abstracts not related to the thesis

1. Mitochondrial DNA^{4977} deletion in human newborn samples
   Kosztolányi G, **Nádasi E**, Melegh B, Morava É Czakó M
   Eur J Hum Genet, 7:P894, A143, 1999 **IF: 2.554**
2. Trans-hexenal, a new naturally occuring carcinogen
   Ember I, Varga C, Pajor L, **Nádasi E,** Varjas T, Nowrasteh G, Csejtey A, Bujdoso L, Rodler I, Kvarda A
   Anticancer Res 24(5D):3479, 2004 **IF: 1.347**
3. The early effect of plant extracts on tumor growth due to carcinogen exposure
   Varjas T, Nowrasteh G, **Nádasi E,** Virág V, Simon A, Gunszt B, Ember I
   Anticancer Res 24(5D):3664, 2004 **IF: 1.347**

Presentations related to the thesis

1. HER2, p53, Fas, FasL, COX2, PGE2S, EGFR expression in breast cancer and in normal peritumoral breast tissue: potential novel risk biomarkers

Nádasi E, Sándor J, Mottolese M, Cianciulli AM, Natali PG, Ember I

October 25-30, 2004 7th International Conference of Anticancer Research, Corfu, Greece

2. COX-2 expression in primary and metastatic lesions of breast cancer patients treated with adjuvant anthracycline-based therapy: prognostic implications

Mottolese M, **Nádasi E,** Botti C, Giannarelli D, Del Monte G, Venturo I, Di Benedetto A, Marandino F, Lopez M, Natali PG

March 27-31, 2004 95th Annual Meeting of AACR, Orlando, Florida, USA

3. [Epidemiology of breast cancer: COX2 as a new prognostic factor]

Nádasi E, Botti C, Giannarelli D, Di Filippo F, Venturo I, Del Monte G, Scordati P, Natali PG, Mottolese M

November 28-29, 2003 1st Conference of the Hungarian Society of Molecular Predictive Epidemiology, Pécs, Hungary

4. [Role of interactions between oncogenes and suppressor genes in the pathogenesis of breast cancer]

Sándor J, **Nádasi E**

November 28-29, 2003 1st Conference of the Hungarian Society of Molecular Predictive Epidemiology, Pécs, Hungary

5. [HER2, p53, Fas and FasL expression in breast cancer: new potencial biomarkers]

Nádasi E, Mottolese M, Natali PG, Varjas T, Ember I.

April 24-25, 2003, NETT 14th Annual Meeting, Hévíz, Hungary

6. HER2 expression and/or gene amplification, p53 nuclear accumulation, Fas-ligand upregulation in breast cancer and autologous peritumoral tissues: diagnostic implications

Mottolese M, **Nádasi E,** Buglioni S, Benevolo M, Cianciulli AM, Merola R, Venturo I, Del Monte G, Giannarelli D, Botti C, Natal, PG

April 5-9, 2003, 94th Annual Meeting of AACR, Toronto, Canada

7. COX2 expression in high risk breast cancer patients treated with adjuvant anthracycline-based therapy

Nádasi E, Botti C, Giannarelli D, Di Filippo F, Del Monte G, Venturo I, Scordati P, Natali PG, Mottolese M

October 27-30, 2002, „From Molecular Oncology to Molecular Therapy" 29th Simposion of the Italian Society of Cancerology, Genova, Italy

8. Immunohistochemical evaluation of HER2, p53, Fas and Fas ligand in breast cancer and multiple adjacent non-neoplastic breast tissues (award of the Organizing Committee)

Nádasi E, Botti C, Natali PG, Buglioni S, Benevolo M, Venturo I, Del Monte G,

Sciarretta F, Giannarelli D, Mottolese M

October 18-22, 2002, 27th ESMO Congress, Nice, France

Presentations not related to the thesis

1. Trans-hexenal, a new naturally occuring carcinogen

   Ember I, Varga C, Pajor L, **Nádasi E,** Varjas T, Nowrasteh G, Csejtey A, Bujdoso L, Rodler I, Kvarda A

   October 25-30, 2004 7th International Conference of Anticancer Research, Corfu, Greece

2. [mtDNA haplogroups in Hungarians: a comparison with three other European populations]

   Nádasi E, Gyűrűs P, Kosztolányi S, Bene J, Fazekas S, Dömösi P, Torroni A, Melegh B

   June 2-4, 2004 Hungary and the Orient – conference on ancient history, Budapest, Hungary

3. [Genetically modified foods]

   Nádasi E.

   October 15, 2003, "Health in the year of the handicapped" Conference, Szombathely, Hungary

4. [Role of the environmental factors in developing inherited diseases]

   Nádasi E.

   March 5-7, 2001 "Mandulavirágzás" Scientific Days, University of Pécs, Pécs, Hungary

5. [Mitochondrial DNA-deletion in a patient with MELAS-syndrome]

   Tóth G, **Nádasi E,** Morava É, Farkas V, Kosztolányi G, Melegh B

   August 25-28, 1999, 2nd Conference of National Association of Human Genetics, Pécs, Hungary

6. [Mitochondrial DNA-polymorphysms in a sample of the Hungarian population]

   Kosztolányi S, **Nádasi E,** Gyûrûs P, Melegh B, Kosztolányi G

   August 25-28, 1999, 2nd Conference of National Association of Human Genetics, Pécs, Hungary

7. Somatic mutations in cadaver brain samples

   Nádasi E, Melegh B, Kosztolányi G

   May 5-9, 1998, 14th International Medical Sciences Student Congress, Istanbul, Turkey

8. [Rothmund-Thomson syndrome in siblings]

   Kosztolányi R, **Nádasi E,** Horváth G, Kosztolányi G, Farkas B

   December 5-6, 1997, Conference of National Association of Dermastologists, Budapest

9. [Somatic mutations in cadaver brain samples]

   Nádasi E, Varjas T, Kovács E, Molnár J, Melegh B, Kosztolányi G

   June 19-21, 1997, Conference of National Association of Pediatricians, Szombathely, Hungary (special award)

10. [Mitochondrial diseases]

    Nádasi E.

    April 17, 1997, Conference of the Veszprém Commitee of the Hungarian National Academy, Gyõr, Hungary

11. [Multiple mitochondrial DNA-deletions in a patient with Brachmann-De Lange phenotype]

    Melegh B, Bock I, **Nádasi E,** Kosztolányi G, Méhes K

    June 13-15, 1996, Conference of National Association of Pediatricians, Budapest, Hungary

12. [Neurological examination of hippocampi taken from newborns with trisomy]

    Nádasi E, Seress L, Tornóczky T, Kosztolányi G

    June 13-15, 1996, Conference of National Association of Pediatricians, Budapest, Hungary

13. [How to use London Dysmorphology Database: Course for Pediatricians]

    Nádasi E.

    April 12, 1996, Pécs, Hungary

14. The early effect of plant extracts on tumor growth due to carcinogen exposure

    Varjas T, Nowrasteh G, **Nádasi E,** Virág V, Simon A, Gunszt B, Ember I

    October 25-30, 2004 7th International Conference of Anticancer Research, Corfu, Greece

15. [2-hexenal: a plant carcinogen with epigenetic effect]

    Nádasi E, Varjas T, Pajor L, Ember I

    May 6-8, 2004 NETT 15th Annual Meeting, Szekszárd, Hungary

16. Present and future of the molecular epidemiology (poster)

    Ember I, Kiss I, Sándor J, **Nádasi E,** Varjas T, Gyöngyi Z, Nowrasteh G, Varga C

    November 20-22, 2003 ”Globalization and health in Europe: harmonising public health practice” EUPHA Conference 2003”, Rome, Italy

17. [New method for investigating tissue samples: chromogenic in situ hybridization]

    Nowrasteh G, Mottolese M, **Nádasi E,** Varjas T, Ember I

    April 24-25, 2003, NETT 14th Annual Meeting, Hévíz, Hungary

18. Investigation of the anticarcinogenic effect of resveratrol in a mouse gene expression model

    Nádasi E, Varjas T, Ember I

June 8-12, 2001, New Anticancer Agents, International Conference Organized and Supported by the International Institute of Anticancer Resarch, Athens, Greece

19. Anticarcinogenic effect of bemitil (an antioxidant compound) in a gene expression model
    Varjas T, **Nádasi E,** Pusztai Z, Ember I, Gyöngyi Z, Durnev A, Kiss I
    June 8-12, 2001, New Anticancer Agents, International Conference Organized and Supported by the International Institute of Anticancer Resarch, Athens, Greece
20. [Role of 2-hexenal, a plant aldehyde in carcinogenesis]
    Varjas T, **Nádasi E.**
    May 9-11, 2001, Conrerence for Young Oncologists, Pécs, Hungary
21. In vivo oncogene expression could be induced with chemical carcinogenes in inbred, sensitive mice and could b used as an early potential biomarker
    Ember I, Kiss I, Gyöngyi Z, Varga C, Perjési P, **Nádasi E**
    November 29 – December 03, 2000 Mouse Models of Cancer, An AACR Special Conference in Cancer Research, San Diego, California, USA
22. Somatic mutations in cadaver brain samples
    Nádasi E, Morava É, Czakó M, Melegh B, Kosztolányi G
    May 29 - June 1, 1999, Congress of the Europen Society of Human Genetics, Geneve, Switzerland
23. [Demonstration of familial t(5;18)(q33;q23) by fluorescent in situ hybridisation (FISH)]
    Nádasi E, Tárnok A, Varjas T, Melegh B, Kosztolányi G
    June 25-27, 1998, Conference of National Association of Pediatricians, Szeged, Hungary
24. [Molecular genetical methods in diagnosing myotonical dystrophia] (award)
    Molnár J, Melegh B, Kis A, **Nádasi E,** Varjas T, Kovács E, Kosztolányi G
    June 19-21, 1997, Conference of National Association of Pediatricians, Szombathely, Hungary
25. [Diagnosing of Prader-Willi syndrome by cytogenetical and molecular genetical methods]
    Varjas T, **Nádasi E,** Kovács E, Molnár J, Kis A, Melegh B, Kosztolányi G
    June 19-21, 1997, Conference of National Association of Pediatricians, Szombathely, Hungary (award)
26. [Molecular biology based method for screening for fragile X syndrome]
    Kovács E, **Nádasi E,** Varjas T, Molnár J, Melegh B, Kosztolányi G
    June 19-21, 1997, Conference of National Association of Pediatricians, Szombathely, Hungary (award)

27. [Evaluation of CTG-triplet expansion in three generations of a family with dystrophical myotonia]

    Molnár J, Kis A, **Nádasi E,** Melegh B, Kosztolányi G

    May 9-10, 1997. Conference of the National Association for Neurogenetics, Szombathely, Hungary

28. [Somatic mutations in cadaver brain samples]

    Nádasi E, Varjas T, Molnár J, Melegh B, Kosztolányi G

    May 9-10, 1997, Conference of the National Association for Neurogenetics, Szombathely, Hungary

29. [Multiple mitochondrial DNA-deletion and persistent hyperthermia in a patient with Brachmann-De Lange phenotype]

    Nádasi E, Bock I, Melegh B, Gáti I, Méhes K

    March 22-23, 1996, 2nd Conference of the association of Young Hungarian Researchers in Pediatrics, Szeged, Hungary